NUTRITION, IMMUNITY AND LUNG HEALTH: TIME TO TAKE CENTER STAGE

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NUTRITION, IMMUNITY AND LUNG HEALTH: TIME TO TAKE CENTER STAGE

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Table of Contents

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EDITORIAL
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Editorial: Nutrition, Immunity, and Lung Health: Time to Take Center Stage

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Editorial on the Research Topic:

Nutrition, Immunity, and Lung Health: Time to Take Center Stage

Globally, we are increasingly becoming subject to a torrent of factors that are taking their toll on lung health. Antibiotic resistance, the emergence of Severe Acute Respiratory Syndrome-2 (SARS-CoV-2) and air pollution are just some of the main drivers behind poor lung health in the modern world. In the past, there has been a generic tendency to focus on other disease entities (Alzheimer's disease, cardiovascular disease, cancer, diabetes, and stroke) and research related to lung health has lagged behind (1).

Chronic respiratory diseases have been overlooked as noncommunicable diseases, yet are one the greatest killers of present day. Chronic obstructive pulmonary disease (COPD) was the third largest global cause of death in 2019 and lower respiratory infections the fourth (2). It is only now, in the era of the coronavirus-19 (COVID-19) pandemic, that an explosion of new research coined the "infodemic" has rapidly shifted the focus towards lung health. This special issue and e-book provides novel input into the field.

In this issue, there are eight papers focusing specifically on nutrition, immunity, and lung health. There has been an emergence of evidence of the immunomodulatory roles of nutrients that could influence respiratory disease risk and progression and their possibilities as adjunctives to conventional treatment regimens. For example, Gozzi-Silva concluded that a range of vitamins (A, C, D, and E), minerals (iron, selenium, magnesium, and zinc), flavonoids, fatty acids, and certain other bioactive compounds have potential roles in reducing the risk of chronic pulmonary diseases and viral infections, due to their anti-inflammatory and antioxidant effects and ability to promote immune responses against pathogens. Singh et al. focused on evidence for nutraceuticals (from human and laboratory studies), concluding that certain vitamins (A, B, C, D, and E), probiotics, bioactive compounds (curcumin, epigallocatechin gallate, resveratrol, and quercetin) and functional foods providing these (e.g., berries and honey) could be beneficial for the immune system but should not be a replacement for a healthy lifestyle when used in supplement form.

Fernández-Lázaro et al. collated evidence on the role(s) of glucans, with particular focus on the protein/polysaccharide AM3, a natural glycophosphopeptical. This has previously been found to modulate the progression of respiratory diseases by regulating innate and adaptive immunity (altering natural killer cell production and interferon secretion and reducing inflammatory cytokine production). Whilst experimental models suggest some promise, clinical trials of these agents are now needed.

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Derbyshire and Calder contributed two articles. The first, "Respiratory Tract Infections and Antibiotic Resistance", focused specifically on the role(s) of vitamin D from a "prehabilitation" stance discussing evidence in relation to how appropriate supplementation could lower the risk of acute respiratory tract infections (ARTIs) and, in turn, play a central role in helping to reduce over-reliance on antibiotics which is contributing to antimicrobial resistance (AMR) (Derbyshire and Calder)—one of the largest pending threats to global health. The second article, "Bronchiectasis—Could Immunonutrition Heave a Role to Play in Future Management?" focused specifically on the condition bronchiectasis—a condition rising in prevalence where the bronchial tubes become permanently widened thus predisposing the lungs to infections. Research is emerging studying the roles of malnutrition and certain nutrients—vitamin D and zinc—and the lung microbiome, but future research is needed to drive advancements in this field forward (Derbyshire and Calder).

Sun et al. discussed the role(s) of gut microbiota in pulmonary disease acting *via* the gut-lung axis. Gentamicin (a broad-spectrum antibiotic) was found to disrupt the gut microbiota which could contribute to enhanced severity of influenza viral infection. Chen et al. from King's College London investigated Vitamin D mechanisms in airway diseases. *In vitro* and *in vivo* work showed that vitamin D appears to increase alpha-1 antitrypsin synthesis by human T cells, and suggests that alpha-1 antitrypsin could represent an intermediate player in some of the immunomodulatory functions of vitamin D. Concentrating on a murine model of tuberculosis (TB). Hayford et al. concluded that omega-3 polyunsaturated fatty acid therapy alongside conventional TB medications could improve anemia of infection and lower cytokine-mediated inflammation. Human studies are now needed to extend these findings.

To conclude, the SARs-CoV-2 pandemic has emphasized the fact that research into chronic respiratory/lung diseases warrants more attention. Whilst it is appreciated that we are living in a world of competing public health priorities, it is now time for lung health to take far greater precedence. Acting directly *via* immunomodulatory effects and indirectly *via* the microbiota, nutrition has a central role to play in preventative healthcare. Just as a Mediterranean diet is advocated for heart health (3), we should now begin to delve deeper into what is warranted to sustain lung health.

AUTHOR CONTRIBUTIONS

ED wrote the first version. PC edited and contributed to this yielding a second version. All authors contributed to the article and authorized the submitted version.

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Conflict of Interest: ED is the Director of Nutritional Insight.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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Gentamicin Induced Microbiome Adaptations Associate With Increased BCAA Levels and Enhance Severity of Influenza Infection

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Involvement of gut microbiota in pulmonary disease by the gut-lung axis has been widely observed. However, the cross-talk messengers between respiratory mucosal immunity and gut microbiota are largely unknown. Using selective pharmacologic destruction of gut microenvironment mouse models, we found gut microbiota displayed significantly lower alpha diversity and relative abundance of bacteria in Gentamicin treated mice. Metagenomic studies revealed functional differences in gut bacteria in altering metabolic profiles in mice blood. Branched-chain amino acids (BCAAs) are the essential factors linked between gut and lung. During this process, selective destruction of gut microbiota by Gentamicin induced high levels of BCAAs, and the high levels of BCAAs impacted the lung immunity against influenza virus. *In vivo*, Gentamicin-treated mice or mice fed with high BCAAs diets displayed reduced survival. At the sites of infection, the number of $CD11b^+Ly6G^+$ cells decreased, and $CD8^+$ T cells increased accompanied by exuberant expression of pro-inflammatory cytokines could result in tissue damage. $CD11b^+Ly6G^+$ cells transplantation conferred remarkable protection from influenza virus infections. *In vitro*, BCAAs promoted bone marrow-derived cells differentiation to dendritic cells. Taken together, these findings demonstrate that Gentamicin induced disruption of the gut microbiota leads to increased BCAA levels that suppress $CD11b^+Ly6c^+$ cell development in association with overactive $CD8^+$ T responses which may contribute to enhanced severity of the viral infection.

Keywords: branched-chain amino acids, gut microbiota, myeloid-derived suppressor cell, influenza, T cells

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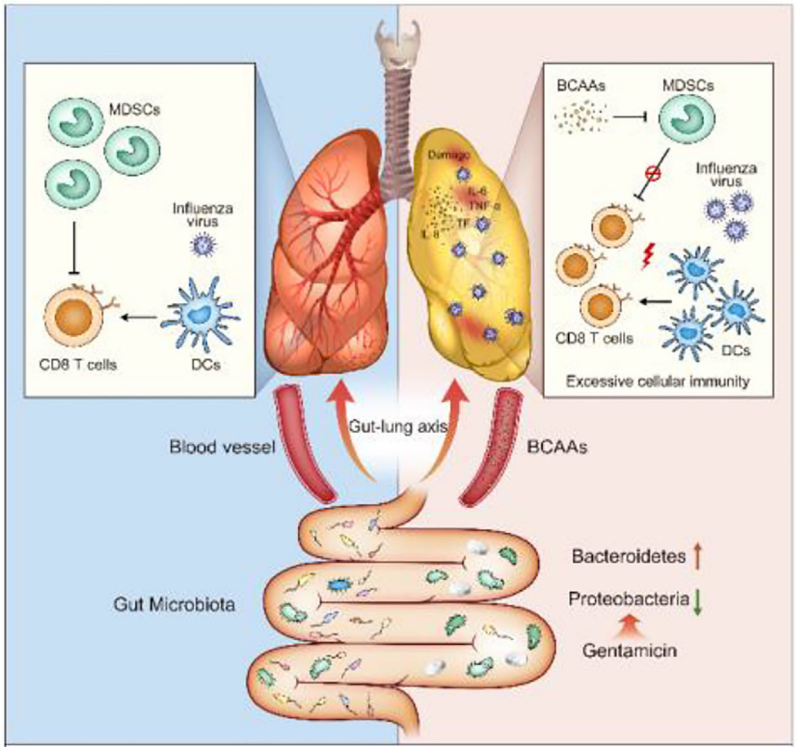

GRAPHICAL ABSTRACT |

INTRODUCTION

The human intestinal tract harbors trillions of microorganisms referred to as the gut microbiota, which fulfils many essential functions, with disruption of the structure of this community leading to dysbiosis (1–3). Many extrinsic factors, especially broad-spectrum antibiotics became a much-debated topic only recently, can easily alter the microbiota by reducing diversity and shifting community composition (4–7). Several mechanisms by which gut microorganisms can modulate the development of metabolic diseases have been reported (8, 9). Interaction between the host *via* metabolic capacities of the gut microbiota with lung immunity is particular interest for infections (2).

Recently, people have reached a greater understanding of gut-lung axis. This axis from the simple fact that gut microbiota can influence lung function and pathogen clearance mediated by metabolites, microbiota produces, or *via* immune cells and immune factors (10). Respiratory tract infectious diseases, such as influenza and pneumonia, result in the death of 2–3 million people annually worldwide. Understanding the mechanisms that mediate cross-talk between the gut microbiota and lung defenses and how this interaction facilitates optimal lung health is of growing interest. Although, mechanistically, this phenomenon remains poorly defined, the existence of the gut-lung axis and its implications in both health and disease could be profoundly important for lung infection.

Influenza virus is the major source of severe viral respiratory infections, and secondary bacterial infections often follow with influenza infection can cause severe morbidity and mortality involving three to five million people each year (5, 11). Thus, antibiotics were usually used during serious virus infections by clinicians. Recent studies highlight the importance of gut microbiota in shaping lung mucosal immunity (6, 12). However, it remains unclear whether all antibiotics cannot be used during serious virus invasion followed secondary bacterial infections, and whether there is a role of gut microbiota in shaping the lung mucosal immune responses by altering metabolic profiles.

Here, we selected several single antibiotics to set up the conditional alteration of gut microbiota animal models instead of the complete depletion of intestinal flora animal models with giving antibiotics cocktail. The studies revealed that selective disruption of gut microbiota diversity only by Gentamicin increased the susceptibility of lung immunity to influenza virus. Gentamicin is used to kill gram-negative bacteria to induce selective pharmacologic destruction of gut microenvironment and altered metabolism. The Branched-chain amino acids (BCAAs) are found in this list, including leucine, valine, and isoleucine. Cell culture studies show that BCAAs are absolutely essential for several immune cells to synthesize protein and proliferation (13). However, many detailed aspects of BCAAs and its effects on immune function have not been studied. In our study, Gentamicin increased branched-chain amino acids (BCAAs) levels to alter mucosal immunity against influenza infection. This impairment displayed by the mouse models strongly suggests that BCAAs are important cross-talk messengers of gut-lung axis to

modulate homeostasis of innate immune response defense against pulmonary infection.

MATERIALS AND METHODS

Mice

All animal studies were conducted in accordance with Beijing Institute of Microbiology and Epidemiology Animal Care and Use Committee guidelines. BALB/c wild type mice (5-week-old, weighting 14–16 g) were obtained from our institute Laboratory Animal Center, Beijing, China. All experimental mice were bred in a specific pathogen-free facility at our institute. Experimental mice were matched for age and sex and cared for according to guide lines of institute. Mice were monitored and weighted at least once daily after initiating infection. Recumbent mice, and mice that lost more than 30% weight, were considered moribund and euthanized.

Virus Infection and Mouse Treatment

Influenza A virus (IAV) A/Puerto Rico/8/1934 (PR8) was propagated in 10-day-old specific pathogen-free chicken embryos. Where indicated, mice were treated pharmacologically by supplementing drinking water with 160 ^{u}/ml Gentamicin, or 5 mg/ml Streptomycin, or 0.05 mg/ml Vancomycin, or 0.5 mg/ml Tinidazole (Sigma-Aldrich, USA) beginning 3 days prior to infection with replenishment every 48 h. Mice were supplied with high-BCAAs diets (5 mg/day, the ration of three amino acids is 1:1:1) and normal diets daily beginning 3 days prior to infection. Infections were performed by applying $10^{3.5}$TCID$_{50}$ influenza PR8 intranasally. All virus infections were performed in BLS-2 laboratory and followed the specific procedures.

16S rDNA Amplicon Sequencing

Total genome DNA was extracted using CTAB/SDS method from colonic contents. DNA concentration and purity were monitored on 1% agarose gels. 16S rDNA/ITS genes of distinct regions (16SV4/16SV3/16SV3-V4/16SV4-V5, ITS1/ITS2, Arc V4) were amplified used specific primer (e.g. 16S V4: 515F-806R, et al.) with the barcode. All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). Samples with bright main strip between 400 and 450 bp were chosen for further experiments. Then, mixture PCR products were purified with Qiagen Gel Extraction Kit (Qiagen, Germany). Sequencing libraries were generated using TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) following manufacturer's recommendations and index codes were added. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. At last, the library was sequenced on an IlluminaHiSeq2500 platform and 250 bp paired-end reads were generated.

Nuclear Magnetic Resonance Metabonomics Analysis

NMR metabonomics analysis was done by Wuhan Anachro. In brief, serum of mice treated with antibiotic and controls were vortexed, aqueous layer was transferred to 0.5 ml 3KDa ultrafiltration filter (Millipore, USA). Fifty microliters H2O and 50 μl DSS standard solution (Anachro, Canada) was added. Samples were mixed well before transfer to 5mm NMR tube (Norell, USA). Spectra were collected using a Bruker AV III 600 MHz spectrometer equipped with an inverse cryoprobe. The first increment of a 2D-1H, 1H-NOESY pulse sequence was utilized for the acquisition of 1H-NMR data and for suppressing the solvent signal. Experiments used a 100 ms mixing time along with a 990 ms pre-saturation (~80 Hz gammaB1). Spectra were collected at 25°C, with a total of 64 scans over a period of 7 min. The collected Free Induction Decay (FID) signal was automatically zero filled and Fourier transform in Processing module in Chenomx NMR Suite 8.1. (Chenomx Inc., Edmonton, Canada). The data were then carefully phased and baseline corrected by experienced technician in Chenomx Processor. All the spectra were referenced to the internal standard, DSS and analyzed by experienced analysts against Chenomx Compound Library. All metabolites' concentration information was exported to excel and normalized by weight across all parallel samples before used in the later on multivariable analysis. PCA and PLS-DA were performed using the pcaMethods bioconductor package (14) and pls package respectively. Plots were made using ggplot2 package (15).

Measurements of Virus Copy and Immune Parameters

Total RNA was obtained from lung tissue with TRIzol reagent (Invitrogen). The cDNA was generated by reverse transcription with commercial PrimeScript RT Master Mix (Takara). Tissue levels of mRNA encoding TNF-α, IL-6, IL-1β, IFN-γ, IL-8, and TF were measured by real-time PCR (LightCycle 480), normalized to levels of mRNA encoding β-actin, and expressed as fold change relative to levels in uninfected wild-type mice. The influenza viral burden in whole lung tissue was determined also by real-time PCR measuring influenza virus membrane protein 1 (M1) copy number. The primer pairs used for real-time PCR were designed using Primer.

	Forward	Reverse
TNF-α	CATCTTCTCAAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC
IL-6	GAACAACGATGATGCACTTG	TGAAGGACTCTGGCTTTGTC
IL-1β	GAACAACGATGATGCACTTG	TTCTTTGGGTATTGCTTGGGA
IFN-γ	CATTGAAAGCCTAGAAAGTCTGAATAAC	TGGCTCTGCAGGATTTTCATG
IL-8	TGGCAGCCTTCCTGATTT	AGGTTTGGAGTATGTCTTTATGC
TF	CAATGAATTCTCGATTGATGTGG	GGAGGATGATAAAGATGGTGGC
M1	AAGACCAATCCTGTCACCTCTG	CAAAACGTCTACGCTGCAGTCC
β-actin	GGAGGGGGTTGAGGTGTT	GTGTGCACTTTTATTGGTCTCAAG

Total IgGs titers of serum specific to PR8 virus were measured by enzyme-linked immunosorbent assay (ELISA). The PR8 virus were freeze thawed repeatedly, then coated in 96-well plates

overnight at 4°C. Serum samples were serially diluted in 2-fold dilutions from 1:10 to 1:20480. The endpoint dilution titer was calculated as the serum dilution resulting in an absorbance reading of 0.2 units above background. Goat anti-mouse IgG-HRP (Sigma, 1:5000) was used as the detection antibodies. The reactions were developed with TMB(3,3',5,5'-Tetramethylbenzidine) and stopped with 2 M H_2SO_4. The absorbance at 450 nm was detected.

Flow Cytometry

Preparation of lung single cells was described by Dr Smiley (16). In brief, cells isolated from lung tissues were ground with glass rod and digested with collagenase, and then single cells were incubated with Fc Block (clone 2.4G2) for 15 min at 4°C, washed 3 times. For enumeration of CD45 cells, macrophages, and neutrophils, pulmonary lymphocytes were stained on ice with anti-CD45-EF450 (cloneRM-5), anti-CD11b-APC (clone M1/70), and anti-Ly6G-FITC (clone 1A8), anti-Gr-1-Alexafluor700 (RB6-8C5), anti-CD3-PerCP-eFlour™-710 (clone17A2), anti-CD4-eFlour®450 (cloneRM4-5), anti-CD8a-PE-Cy7 (clone53-6.7) (eBioscience, USA). Data were gated for forward scatter/side scatter and collected on an FACSCanto II (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Spleen $CD11b^+Ly6G^+$Cells Isolation and Adoptive Transfer

The method for $CD11b^+Ly6G^+$cells purification is supplied by Dr Tsukasa Seya's team (17). $CD11b^+Ly6G^+$ cells were isolated from a single cell suspension from the spleen of wild-type mice by using a biotin-conjugated anti-Ly6G monoclonal Ab (1A8) (Biolegend, San Diego, CA, USA) and Streptavidin Microbeads (MiltenyiBiotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. In these purification steps, two rounds of positive selection were performed to increase purity. We routinely prepared $Ly6G^+$cells at >95% purity and almost 100% of $Ly6G^+$ cells expressed CD11b. Isolated $CD11b^+Ly6G^+$cells were resuspended in PBS, and then adoptive transferred intravenously into recipient mice (5×10^5 viable cells/mouse), which were challenged with $10^{3.5}TCID_{50}$ (mice treated with Gentamicin 3 days) influenza 3 days before $CD11b^+Ly6G^+$ cells transfer.

$CD11b^+Ly6G^+$ Cells Induction From Bone Marrow Stromal Cells

The method of bone marrow stromal cells preparation is described by Dr Dina Sabry (18). In brief, Tibia and fibula bone marrow was flushed out with phosphate-buffered saline (PBS) containing 2 mM EDTA for isolation and culture. Then, the sample was layered carefully on Ficoll-Paque (Gibco-Invitrogen, Grand Island, NY), and keep the mononuclear cell layer for the next study. Cells were stimulated *in vitro* in bulk culture with granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin-4 (IL-4). In brief, thirteen days after initiation of culture, the culture was replenished with an equal volume of medium containing 50 ng/ml recombinant human GM-CSF every other day. After 5 days of culture, the cultures were replenished with an equal volume of medium containing 50 ng/ml recombinant human IL-4 and/or different concentration of BCAAs (5 and 1 μg/μl, the ration of three amino acids is 1:1:1) (Qianrun Inc. China) every other day. After 3 days of culture, cells were harvested and evaluated by Western-blot and Flow cytometer.

Western-Blot

Bone marrow stromal cells were cultured with GM-CSF/IL-4 and different concentration of BCAAs/rapamycin (Sigma-Aldrich, USA). Cells were harvested and analyzed by 8% SDS-PAGE. Samples separated in the gel were electro transferred to a PVDF membrane (GE). This step was followed by the antigen-antibody reactions. Rabbit anti-mTOR hyper-immune sera was used as the detecting antibody, and horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) served as the secondary antibody (Cell Signaling Technology Inc). Following the addition of the substrate diaminobenzidine, the specific protein bands were revealed.

Histology

Lung tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. The pathological foci in each section were evaluated. (i.e. areas with large numbers of inflammatory cell infiltration accompanied by evidence of edema of submucosa). Representative photomicrographs depict × 100 magnification.

Statistical Analysis

Statistical analyses were performed using the program Prism 5.0 (GraphPad Software, Inc., La Jolla, California, USA). Values are expressed as mean ± SD. Data were analyzed by unpaired Student's t-test (normal distribution) or one-way ANOVA followed by Dunnett's multiple comparison tests. Survival data were analyzed by log rank tests. Statistical analysis of microbiota data was performed in Rhea (19). EzTaxon (20) was used for the identification of OTUs showing significant differences ($p<0.05$) in relative abundances between feeding groups. MetPA. Pathway analysis was launched by MetaboAnalyst 3.0 using identified differential metabolites (variables with VIP of >1 or $P< 0.05$ in one-way ANOVA). $p<0.05$ was considered to be statistically significant.

RESULTS

Conditional Alteration of Gut Microbiota Increased Susceptibility to Influenza Virus Infection in Lung

To investigate the role of gut microbiota diversity altered by different antibiotics during influenza virus infection, mice were treated with antibiotics (Streptomycin, Vancomycin, Tinidazole, and Gentamicin, all antibiotics cannot be absorbed easily by intestine) separately, and then intranasal challenged with sublethal dose ($10^{3.5}TCID_{50}$) Influenza A virus (A/Puerto Rico/8/1934, PR8). This single antibiotic treated animal models are totally different from mice giving antibiotics cocktail for depletion of intestinal flora completely. We found only

Gentamicin can cause mice dead (**Supplementary 1-1**, **Figure 1A**). Control mice readily survived sublethal dose intranasal challenge with $10^{3.5}$TCID$_{50}$ influenza virus PR8 (**Figure 1A**). Parallel evaluations of body weight changes over the course of infection suggested that the control mice experienced less severe disease than did mice treated with Gentamicin (**Figure 1A**). This susceptibility correlated with increased virus titers in the lung. Meanwhile, we also challenged Gentamicin treated mice with sublethal dose of H5N1 influenza virus, and then measured susceptibility to this subtype influenza virus. We observed that Gentamicin treated mice also succumbed to sublethal dose of H5N1 influenza challenge (data not shown). Notably, susceptibility to influenza virus infection did correlate with gut microbiota diversity. In our study, significant differences were observed in the diversity and composition of gut microbiota in antibiotic treated mice (**Supplementary 2-1**). Analysis of mice gut microbiota showed significantly lower alpha diversity and relative abundance ($p<0.01$) of bacteria in antibiotic treated mice(data not shown). Mice treated with Gentamicin shifted intestinal community by increasing *Bacteroidetes* and decreasing *Proteobacteria* (**Supplementary 2-1**). To further investigate the impact of gut microbiota mediated protection from influenza virus infection, we evaluated pro-inflammatory responses and tissue damage in the lung. We measured inflammation markers and pathology in the lung of mice treated with Gentamicin and control mice at day 8 after inoculation of sublethal dose of influenza virus PR8. Consistent with our previous discoveries of virus titers (**Figure 1B**), Similar trends were observed for markers of inflammation, including levels of mRNA encoding IFN-γ, TNF-α, IL-6, IL-1β, IL-8, and TF. Following levels of inflammatory markers detection, we also assayed the pathological score after the virus infection. In Gentamicin treated mice, the cellular infiltrates tended to be medium to large and were frequently associated with large areas of pulmonary edema, whereas the foci in the control mice tended to be small to medium, with little evidence of edema (**Figure 1C**). Pathological scoring revealed significant differences in the number and quality of pulmonary foci showing high degrees of edema.

CD8$^+$ T cells can facilitate the major protection against influenza strains, thus, cellular immunity to influenza might be predicted to exacerbate susceptibility to respiratory infection disease (21–23). However, depletion of CD8$^+$ T cells diminished the protection against influenza virus infection (24). Thus, maintaining the appropriate levels of T cell responses is very important during virus infection. To test the role of immune cells in defense against virus-induced respiratory susceptibility, we administered Gentamicin to naive mice prior to infection with PR8 influenza, and then tested immune cell responses. We observed that the number of pulmonary CD8$^+$T cells (**Figure 1D**, **Supplementary 1-2A**) increased, CD11b$^+$Ly6G$^+$ cells (**Figure 1D**, **Supplementary 1-2B**) decreased and dendritic cells (DCs) (**Figure 1D**, **Supplementary 1-2C**) increased significantly in Gentamicin-treated mice. CD11b$^+$Ly6G$^+$ cells are identified as granulocytic myeloid derived suppressor cells (G-MDSCs). Just like in tumor

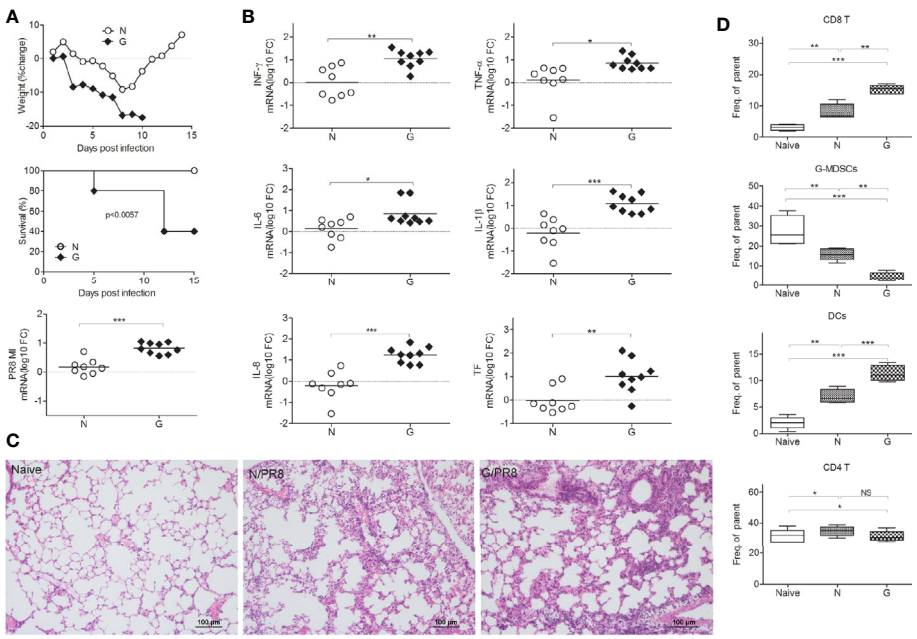

FIGURE 1 | Gut microbiota influences susceptibility to influenza virus infection in lung. Gentamicin treated mice and control mice were inoculated with a sublethal dose ($10^{3.5}$TCID$_{50}$) of influenza PR8, and monitored survival and weighted mice every day. On day 8 after the inoculations, mice were euthanized, **(A)** percent body weight change, survival, lung virus titer and **(B)** lung levels of mRNA encoding IFN-γ, TNF-α, IL-6, IL-1β, IL-8, and TF were measured (n=8-10 per group). Gut microbiota helped to reduce pathology during infection **(C)**, naïve mice (Naïve), mice infected with virus (N/PR8), mice treated with Gentamicin and then infected with virus (G/PR8). **(D)** CD8$^+$ T cells, CD11b$^+$Ly6G$^+$ cells (G-MDSCs), CD4$^+$ T cells, and DCs were evaluated by Flow cytometry. *<0.05, **p<0.01, ***p<0.001. N, none, G, Gentamicin.

mouse models, we believe that the abundance of $CD11b^{+}Ly6G^{+}$ cell is the important role of regulation $CD8^{+}$ T cell responses during acute virus infections. This subtype myeloid-derived suppressor cells (MDSCs) are responsible for the suppression of T cell-mediated immunity that induced tissue damage.

Gentamicin Treatment Altered BCAAs Levels

To investigate the importance of diversity of gut microbiota and metabolic profile during virus infections, the High-throughput 16S rDNA gene amplicon analysis and nuclear magnetic resonance (NMR) metabonomics analysis were used. Significant differences were observed in the diversity and composition of gut microbiota in Gentamicin treated mice. Analysis of mice gut microbiota showed significantly lower alpha diversity and relative abundance ($p<0.01$) of bacteria in Gentamicin treated mice (**Supplementary 2-1**), such as *Bacteroidetes*, *Proteobacteria* and so on. Metagenomic studies revealed functional differences in gut bacteria in altering metabolic profiles in mice blood (**Supplementary 2-1**). **Supplementary 2-2** indicates that 21 compounds were identified in Gentamicin treated samples (n=7), including 7 amino acids, glucose, choline, lactate and so on. After filtering the metabolites [variable importance in projection (VIP) value of >1 or P< 0.05 in intergroup comparisons by one-way analysis of variance (ANOVA)], hierarchical clustering showed different metabolic signatures between groups (**Figure 2A**). A metabolic pathway analysis (MetPA) of altered metabolites was performed by using the MetaboAnalyst 3.0 online tool (25), revealing the change of 16 compounds metabolism pathway to be pronounced (**Figure 2B**). The Branched-chain amino acids (BCAAs) are found in this list, including leucine, valine, and isoleucine. To further investigate which compound plays the key role defense against influenza infection, we assayed some important compounds *in vivo*. We found the function of BCAAs during influenza virus infection. BCAAs are known to play positive roles during host immunity to bacterial infection (13). Indeed, in our studies, mice administered with Gentamicin markedly increased levels of BCAAs in blood, whereas these mice succumbed to sublethal dose influenza virus challenge (**Figure 1B**). So we believe BCAAs can "modulate" immune homeostasis in different models. To assess the role of BCAAs played during virus infection, mice were supplied with high-BCAAs diets and normal diets, and then intranasally challenge with sublethal dose influenza virus. Consistent with our observation we did in Gentamicin treated animal model, we also observed that mice succumbed to virus challenge when supplied with high-BCAAs diets (**Figure 2C**), and the number of pulmonary $CD8^{+}$ T cells

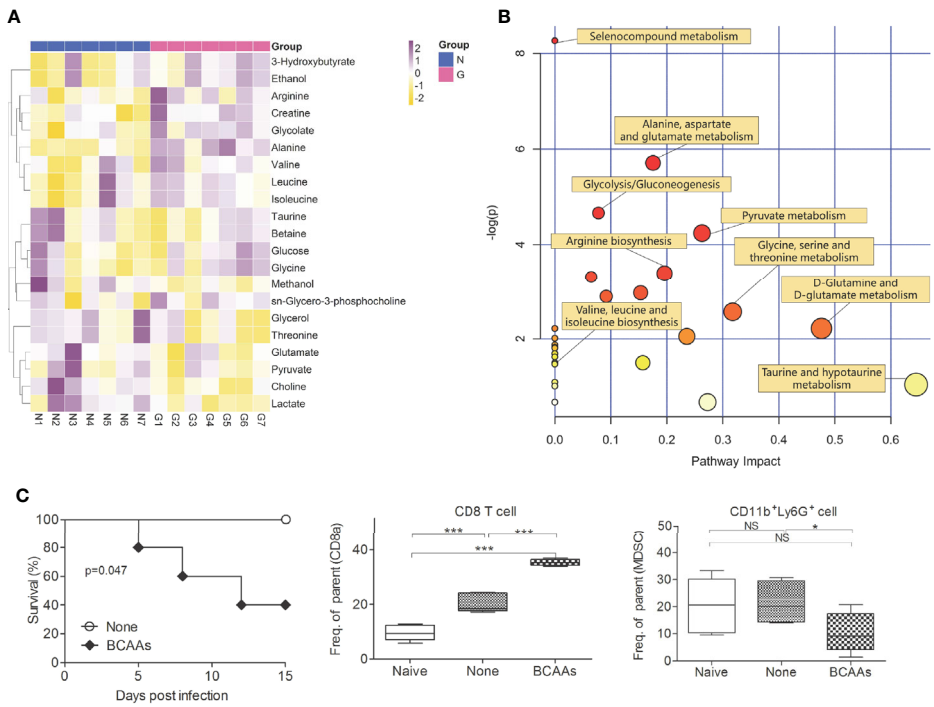

FIGURE 2 | Gentamicin-induced alteration of metabolomics profiles. Serum (n=7) were isolated form mice treated with Gentamicin (G) and none (N), and then analyzed the metabolomics profile. Hierarchical clustering of identified differential metabolites (variables with VIP of >1 or P< 0.05 in one-way ANOVA) **(A)** were shown by heatmap. Each row shows relative ion intensity for a specific metabolite after mean centering and unit variance scaling of the data. Each column shows the serum metabolic profiles of the two groups. **(B)** MetPA. Pathway analysis was launched by MetaboAnalyst 3.0 using identified differential metabolites (variables with VIP of >1 or P< 0.05 in one-way ANOVA). The color and size of each circle are based on its P-value and pathway impact value, respectively. **(C)** Survival, $CD8^{+}$T cells and $CD11b^{+}Ly6G^{+}$ cells (G-MDSCs) were shown in mice supplied with high-BCAAs diets, and then intranasally challenge with sublethal dose influenza virus. *<0.05, **p<0.01, ***p<0.001. NS, no significant.

increased and $CD11b^+Ly6G^+$ cells (G-MDSCs) decreased significantly. Thus, gut microbiota mediated high level of BCAAs may have influenced $CD11b^+Ly6G^+$ cells' function, and the disruption of immune balance between $CD8^+$ T cells and $CD11b^+Ly6G^+$ cell exacerbated susceptibility to respiratory infection disease.

BCAAs Levels Mediated Differentiation of Bone Marrow-Derived Cells (BMDCs) *via* mTOR Contributes to $CD11b^+Ly6G^+$ Cells Exhausting

To investigate this possibility, we isolated BMDCs and examined the proliferation under different concentration of BCAAs *in vitro*. We found that cellular morphology was different after BMDCs were treated with BCAAs (**Figure 3A**). The flow cytometry was used to identify the percent of DCs, macrophages and granulocytes under different concentration of BCAAs. With the same induction method, we found only DCs increased (**Figures 3B, C**) when BMDCs were treated with BCAAs. These results were also found *in vivo* when mice treated with Gentamicin (**Figure 1D**). All findings suggest that BCAAs were important for functional MDSCs differentiation. BCAAs involve activation of mammalian target of rapamycin (mTOR) pathway, which mediates multiple cellular functions, including controlling the maintenance and function of T-reg cell in the periphery (26, 27). Cell culture studies show that BCAAs are absolutely essential for several immune cells to synthesize

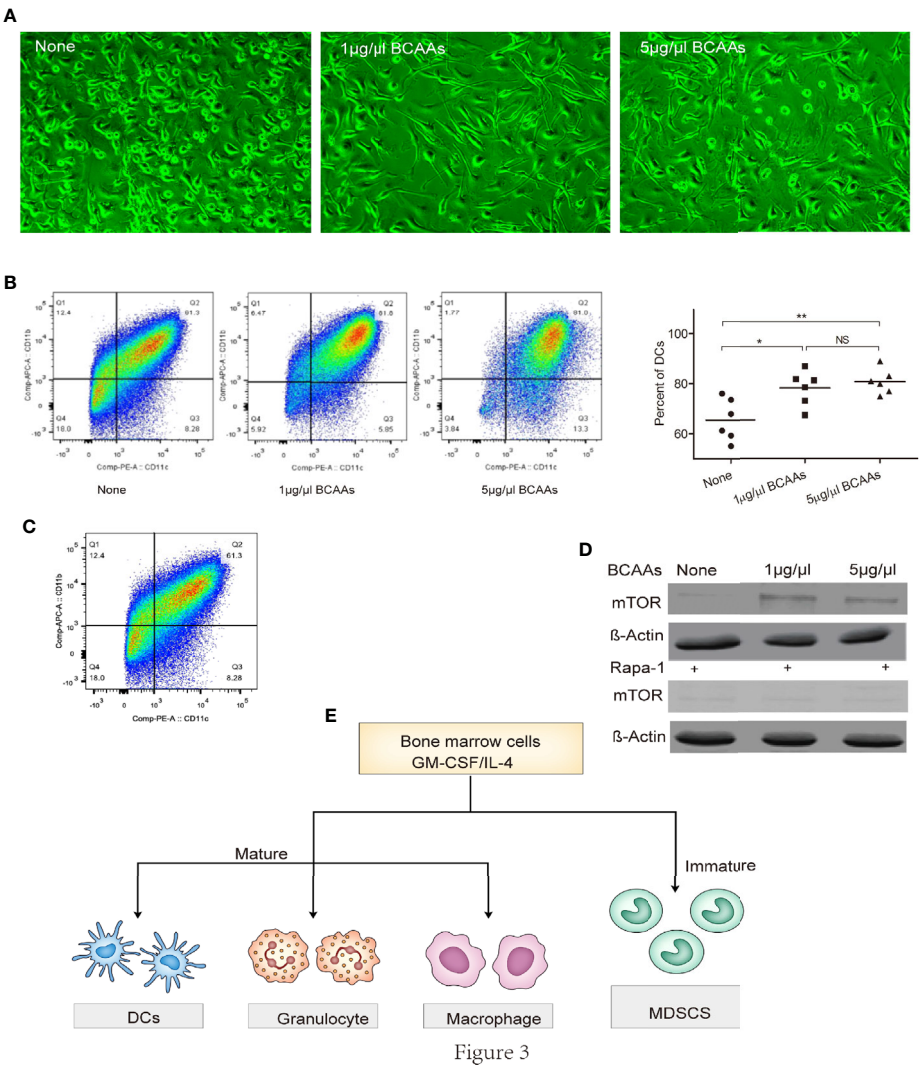

Figure 3

FIGURE 3 | BCAAs are essential to promote BMDCs differentiation into DCs. Bone marrow-derived cells were isolated and examined the differentiation or proliferation under different concentration of BCAAs *in vitro*. **(A)** Cell morphology under microscope, **(B)** Percent of DCs, **(C)** $CD11b^+Ly6G^+$ cells induction from BMDCs, **(D)** Western-blot of mTOR, **(E)** Mechanism of BMDCs differentiation. N, none, G, Gentamicin. All assays were repeated at least three times. *<0.05, **p<0.01, ***p<0.001. NS, no significant.

protein and proliferation. To investigate the mechanism involved in BCAAs induced functional MDSCs differentiation, we evaluated mTOR expression during $CD11b^{+}Ly6G^{+}$ cells proliferation or differentiation. We found that the mTOR expression in BMDCs were promoted by BCAAs and inhibited by rapamycin (**Figure 3D**). We believe that BCAAs impacted mTOR expression to induce BMDCs to differentiate into mature DCs in pulmonary microenvironment. **Figure 3E** depicts the proliferation and differentiation of BMDCs. Immature myeloid cells are part of the normal process of myelopoiesis, which takes place in the bone marrow and includes monocytic MDSCs (M-MDSCs) and G-MDSCs. Normally, the ability to differentiate into mature DCs and macrophages has been shown to be restricted to M-MDSCs. Our results suggested that BCAAs levels with pulmonary microenvironment induced BMDCs to differentiate into DCs maybe through M-MDSCs, which will result in G-MDSCs reduction.

Adoptive Transfer With $Ly6G^{+}CD11b^{+}$ Cells That Confer Protection Against Pulmonary Influenza Virus Infection

We used virus infection model for our research, one very important immune readout is viral specific IgGs responses. To assess whether viral specific IgGs titers also played an important role in mice treated with Gentamicin, serum samples were collected on day 8 after infection. We did not find any differences in total IgG titers between mice treated with/without Gentamicin (**Figure 4A**).

To investigate whether $CD11b^{+}Ly6G^{+}$ cells could suffice to protect against acute influenza virus infection, infected mice were transferred with $CD11b^{+}Ly6G^{+}$ cells. Before the cell transfer, we

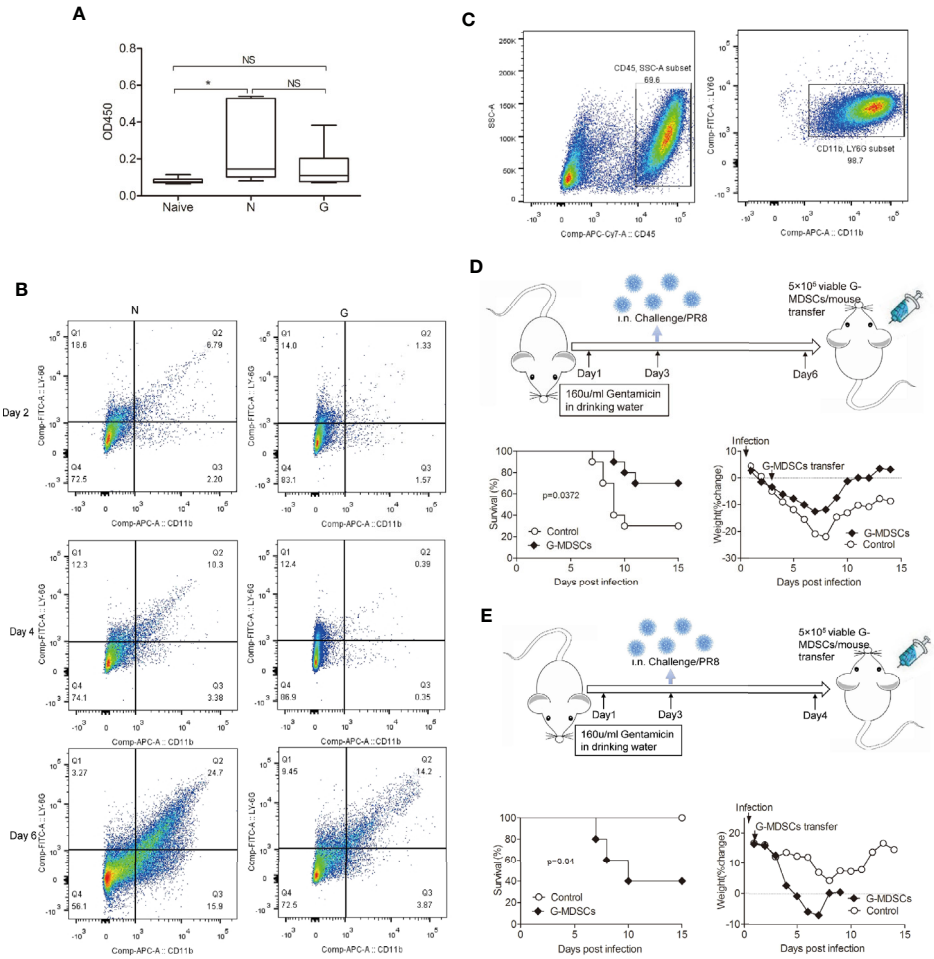

FIGURE 4 | Inhibition of exuberant T cell responses and inflammation by purified $CD11b^{+}Ly6G^{+}$ cells. **(A)** Wild-type mice were injected with anti-Ly6G-specific mAb and anti-CD11b-specific mAb, most mice died with influenza virus challenge. **(B)**Time course of $CD11b^{+}Ly6G^{+}$ cell numbers in Gentamicin treated mice after challenged with influenza. **(C)** $CD11b^{+}Ly6G^{+}$ cells were purified from spleen single cells. **(D)** Mice treated with Gentamicin were transferred with $CD11b^{+}Ly6G^{+}$ cells on day 3 after sublethal dose influenza virus infection, survival and percent body weight changes. **(E)** Same mice were transferred with $CD11b^{+}Ly6G^{+}$ cells on day 1, survival and percent body weight change. N, none/PR8, G, Gentamicin/PR8. All assays were repeated 2 times *<0.05, **p<0.01, ***p<0.001. N, none; G, Gentamicin; NS, no significant.

evaluated the time course of $CD11b^+Ly6G^+$ cells accumulation during influenza infection in Gentamicin-treated mice (**Figure 4B**). We found that the number of $CD11b^+Ly6G^+$ cells kept lower in Gentamicin-treated mice in comparation with normal controls, and bottom on day 3 or on day 4. We believe day 3 or day 4 is the best window period for cell transplantation. So, we purified $CD11b^+Ly6G^+$ cells (**Figure 4C**) according to the method supplied by Dr Tsukasa Seya's team for the next step. Initially, wild-type mice were treated with Gentamicin and then challenged with sublethal dose of influenza virus intranasally. After 3 days, we employed an adoptive transfer strategy in which $CD11b^+Ly6G^+$ cells were purified from spleen, and then injected 5×10^5 viable cells intravenously into mice. In comparison with mice transferred with microbead only, mice transferred with $CD11b^+Ly6G^+$ cells displayed modest but significant protection ($p<0.05$) after challenged with sublethal dose of influenza, as evidenced by both a 2-day prolongation in median survival time and an increase in the overall survival rate to 40% (**Figure 4D**). To further investigate whether the protection conferred by adoptive transferred with $CD11b^+Ly6G^+$ could be used for acute virus infection anytime or any condition, we transferred 5×10^5 viable cells intravenously into recipients without Gentamicin treatment. Control mice received microbeads alone. We observed opposite results (**Figure 4E**), which suggested that $CD11b^+Ly6G^+$ cells could suffice to protect against acute virus infection when the homeostasis of immune cells was disrupted.

DISCUSSION

Antibiotics have been proposed as supplements in re-feeding program for malnourished children. A review of pediatric literature showed that growth promotion by antibiotics (7), when it was observed, was mostly mediated by its anti-infective properties. Despite the widespread use of antibiotics as growth promoters in animal rearing, the available evidence again points to the suppression of infections as the underlying mechanism (4). The potential clinical relevance of the devastating effect of antibiotics on the gut microbiota has become a much-debated topic only recently. Therefore, scientists set antibiotics treated animal models to address the influence of gut microbiota to lung, most of them gave antibiotics cocktail to mice to kill bacteria almost completely (12). We were curious if all antibiotics could induce disruption of the structure of gut microbiota leading to dysbiosis. Here, we treated mice with different kind of antibiotics to address it. All antibiotics can alter the diversity of gut microbiota definitely. However, only Gentamicin treated mice increased susceptibility to influenza virus infection. Gentamicin was used as an oral medicine for diarrhea worldwide more than 10 years ago. Usually, clinicians use it as a very safe antibiotic only because it can't be absorbed by intestine. But the FDA reported in 2019 that influenza infection was found among people who take Gentamicin, especially for people who are 2–9 years old. In our study, mice treated with Gentamicin shifted intestinal community by increasing *Bacteroidetes* and decreasing *Proteobacteria*. This feature of intestinal commensal flora altered by Gentamicin is very special for influenza infection.

Because we gave mice Gentamicin only 3 days, it was difficult that gut microorganisms could colonize in respiratory tract in this short period (28). We concluded by drawing attention to the interactive messengers between gut and lung which were influenced by altered gut microbiota. In the present study, we demonstrated that BCAAs control the maintenance and function of $CD11b^+Ly6G^+$ myeloid-derived suppressor cells in the lung tissue, and BCAAs may promote mTOR activity during DCs differentiation from BMDCs. Although $CD11c^+/CD11b^+$ DCs express high major histocompatibility class II (MHC-II), these cells still can present endogenous antigens to T cells by MHC-I/MHC-II cross-presentation. Ikeda et al. find that BCAAs are essentially required to maintain expansion and suppressive capacity of Treg cell *via* mTOR, which regulate excess immune responses (27). In tumor models, $CD11b^+Ly6G^+$ myeloid-derived suppressor cells also be considered as a regulatory cell subtype to suppress T cell responses (29, 30). Thus, it looks like that BCAAs are important to modulate immune homeostasis. Usually, people take BCAAs to make their muscle strong. However, during epidemics of seasonal influenza, people should take BCAAs discreetly.

Inflammatory responses are initiated in response to pathogen infection, contributing to protective host immunity. However, failure to control exuberant expression of pro-inflammatory cytokines can result in tissue damage (31, 32). In our study, we detected high levels of pro-inflammatory cytokines expression and inflammatory foci with various degrees of lung damage in virus infected mice treated with Gentamicin. Elevated proliferation of the high levels of $CD8^+$ T cells and low levels of $CD11b^+Ly6G^+$ myeloid-derived suppressor cells have been associated with enhanced inflammation (33). MDSCs are an immunosuppressive subset of cells that arise from myeloid progenitors (34, 35), and expressing both $CD11b^+$ and $Ly6G^+$ surface markers cell subtype represents a population of tumor-supporting myeloid cells (36). $CD11b^+Ly6G^+$ cells were shown to produce reactive oxygen species/reactive nitrogen species including hydrogen peroxide and peroxynitrite (PNT). PNT is responsible for the suppression of T cell-mediated immunity that enable tumor cells to escape tumor antigen-specific CTLs (37, 38). However, recent studies have revealed that functionally polarized $CD11b^+Ly6G^+$ cells that means this cell subtype has both a positive and negative impact on tumor growth (39, 40). Meanwhile, the function of MDSCs during acute infection still keep unknown. Thus, a full understanding of the phenotype and function of each of these cell populations is required in order to understand the mechanisms that clear pathogens, prevent systemic spread, and reduce immune-mediated tissue damage.

Because we have no way to decrease the levels of BCAAs in our animal models, we presume if we can use $CD11b^+Ly6G^+$ cells transplantation which levels were influenced by BCAAs instead. Our observations demonstrated that adoptive transfer with $CD11b^+Ly6G^+$ cells that conferred remarkable protection from

influenza virus infections. Under certain conditions, $CD11b^+Ly6G^+$ cells can dampen the inflammatory response through inhibiting T cell responses (41), and $CD11b^+Ly6G^+$ cells will be used clinically as an anti-inflammatory cell subtype, especially during acute virus infection.

Studies over the past a few years have revealed remarkable interplay between microbiota, immunity, and infection. This report extends those connections by demonstrating that gut microflora leading to changing metabolic characterization can critically influence the homeostasis of immune response against acute virus infection.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by Beijing Institute of Microbiology and Epidemiology Animal Care and Use Committee guidelines.

AUTHOR CONTRIBUTIONS

All authors discussed the results and implications of the manuscript. HW and DL conceived the study, supervised the project, analyzed data, and wrote the paper. YS, ZH, JL, SG, SY, TL, NN, LX, LZ, FC, ZL, and JW performed experiments and analyzed data. DL advised on statistical evaluations. YS and ZH contributed equally to this work. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.608895/full#supplementary-material

Supplementary 1-1 | Different antibiotics treatment followed by influenza virus infection. Antibiotics treated mice and control mice were inoculated with a sublethal dose ($10^{3.5}TCID_{50}$) of influenza PR8. On day 8 after the inoculations, mice were euthanized, **(A)** Survival, **(B)** Percent body weight change.

Supplementary 1-2 | Flow cytometry analysis of lung cells of mice treated with Gentamicin and then infected with influenza. Gentamicin treated mice and control mice were inoculated with a sublethal dose ($10^{3.5}TCID_{50}$) of influenza PR8. On day 8 after the inoculations, mice were euthanized, **(A)** $CD8^+$ T cells, **(B)** $CD11b^+Ly6G^+$ cells, and **(C)** DCs. N, none/PR8, G, Gentamicin/PR8.

Supplementary 2-1 | Gentamicin-induced alteration of colonic microbiota profiles. **(A)** Tags and outs **(B)** Relative abundance in phylum levels. **(C)** Extended error bar plot showing the two bacterial genera with a significant difference (T-test; $P< 0.05$) in proportions of at least 1% between samples of control group (N) and samples of Gentamicin-treated group (G). One genera(*Bacteroidetes*)is over abundant within the colonic microbiotas collected from mice treated with Gentamicin compared to those collected from control mice, genera abundance (left) and 95% confidence intervals (right), were detected in 7 Getamicin-treated mice. Number of mice: Control, 7; Gentamicin treated group, 7. N, none/PR8, G, Gentamicin/PR8.

Supplementary 2-2 | Metabolite assignments and chemical shifts of distinguishable peaks.

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Respiratory Tract Infections and Antibiotic Resistance: A Protective Role for Vitamin D?

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Upper and lower respiratory tract infections are among the most common infections globally, and in the United Kingdom, they account for about half of all oral antibiotics prescribed. Antibiotic overuse and the emergence of "superbugs" that are resistant to their effects is a global problem that is becoming a serious concern. Considering this, the potential role of immunonutrition as a "prehabilitation" in helping to tackle bacterial infections and reduce over-reliance on antibiotic usage is gaining interest. This narrative mini-review summarizes current knowledge on the roles of certain nutrients in helping to modulate immune function, with particular focus on vitamin D. Vitamin D supplementation appears to reduce the risk of acute respiratory tract infections and thus could have a valuable role to play in reducing over-reliance on antibiotics. Investment in high-quality trials is needed to further explore this field.

Keywords: respiratory tract infections, antibiotic resistance, vitamin D, immunonutrition, prehabilitation

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INTRODUCTION

There has been an upsurge of novel bacterial, viral, and fungal respiratory pathogens that are becoming increasingly challenging to treat, with respiratory tract infections (RTIs) being exacerbated by antibiotic resistance of Gram-positive and Grain-negative bacteria (1). Acute respiratory tract infections (ARTIs), which include upper respiratory tract infections (URTIs), are, among adults, the most common cause of antibiotic prescription (2). In the United Kingdom, an examination of over eight million patient records from 587 general practices showed that URTIs accounted for around 31% of oral antibiotic prescriptions and lower respiratory tract infections (LRTIs) accounted for around 19% (3).

The very first antibiotic, salvarsan, was developed in 1910 while penicillin discovery by Alexander Fleming followed in 1928 (4). Multiple antibiotics have been discovered since then, but now, after about 100 years of the "antibiotic era," fewer new antibiotics are being identified and significant antibiotic resistance has emerged (4). The World Health Organization considers that the unprecedented use of antibiotics and subsequent antimicrobial resistance (AMR) is currently one of the largest threats to global health, food security, and human development (5, 6).

In 2016, ARTIs were responsible for ∼2.38 million deaths globally (7, 8). Within the European Union, 25,000 people have been estimated to die annually because of AMR with resultant societal costs of around 1.5 billion euros annually (9). By 2050, it has been estimated that some 10 million people globally could die annually as a result of AMR—with 390,000 Europeans estimated to be affected and even larger proportions of Asian (4,730,000) and African (4,140,000) populations (6).

It has been further predicted that standard antibiotic treatments may no longer work, subsequently making infections more difficult to treat and control (10).

Given the high prevalence of ARTIs coupled with rising rates of AMR, novel approaches are needed for the future. The concept of "prehabilitation," including the role of immunonutrition, could play a pivotal role in helping to both prevent and offset RTIs should these occur. Prehabilitation has been well defined elsewhere as: "*interventions that can help to improve patient's health in advance of being exposed to a physiological stressor so they are then better able to cope with that stress*" (11). This narrative mini-review describes how immunonutrition could become a valuable tool in conventional medicine. It focuses on ARTIs and vitamin D, for which there is an expanding body of evidence.

NUTRITION, INFECTION, AND IMMUNITY

The roles of nutrients in supporting the function of the immune system are numerous and varied, with an adequate and balanced supply of nutrients being required if a suitable immune response is to be mounted (12). The immune system protects the body against infectious agents and is composed of innate responses—the body's first lines of defense—and adaptive responses that generate immunological memory (13). It is known that a bidirectional relationship exists between nutrition, infection, and immunity with changes in one impacting on each of the others (14). Micronutrients (vitamins and minerals) have extended roles influencing and supporting every stage of the human immune response (13). Subsequently, deficiencies in one or more micronutrients can affect both innate and adaptive immunity, resulting in immunosuppression and exacerbating susceptibility to infections (13).

A host of nutrients have been implicated as being essential for immunocompetence, including vitamins A, B2, B6, B9 (folic acid), B12, C, D, E, and iron, zinc, selenium, copper, and magnesium (14). Vitamins A, C, D, E, and zinc are important for the structural and functional integrity of the body's external and mucosal barriers to invading pathogens (15). Cellular processes of both innate and adaptive immunity, such as cell differentiation and proliferation, phagocytosis, respiratory burst, killing activity, cytokine production, and antibody production are all dependent on suitable amounts of vitamins A, D, C, E, B6, and B12, folate, iron, zinc, copper, selenium, and magnesium (15).

This mini-review focuses on vitamin D, due to the growing body of evidence favoring a role for vitamin D in preventing ARTIs. Vitamin D augments host barrier epithelial integrity by reinforcing intercellular junctions (16). It has also been found to trigger antimicrobial peptide production, which exhibits direct pathogen-killing capacity (17). The vitamin D receptor is expressed on many immune cell types including B-cells, T-cells, and antigen-presenting cells (18–20). Furthermore, some immune cell types, including macrophages and dendritic cells, can synthesize the active form of vitamin D, 1,25-dihydroxyvitamin D3 (21). These two observations suggest a high importance for vitamin D within the immune system. Indeed, vitamin D deficiency results in impaired localized innate immunity and a defective antigen-specific cellular immune response, correlated with a higher susceptibility to infections (22). Vitamin D metabolites have also been found to influence the expression and secretion of pro-inflammatory chemokines and cytokines (23), and vitamin D promotes the production of anti-microbial peptides such as cathelicidin (21, 24).

An established body of evidence now shows that 1,25-dihydroxyvitamin D3 influences endothelial membrane stability and acts on multiple parts of the innate and adaptive immune responses (21). Low levels of 1,25-dihydroxyvitamin D3 correlate with an increased risk of developing several immune-related disorders including respiratory infection and COVID-19 (21). Vitamin D has further been found to be involved in pulmonary angiotensin-converting enzyme 2 expression and has the ability to reduce lung surface tension in COVID-19 (25). Other work suggests that vitamin D may induce progesterone-induced blocking factor and exert inhibitory effects on inflammation including the cytokine IL-6, which tend to be elevated in COVID-19 (26).

VITAMIN D AND RTIs

A growing number of studies have investigated the role vitamin D on the occurrence of ARTIs. **Table 1** summarizes evidence from meta-analyses and **Table 2** summarizes evidence from RCTs, published in the last 5 years with a focus on adulthood, although some meta-analyses included extended age ranges.

Two meta-analyses focused on observational research (29, 30) and four focused on evidence from RCTs (27, 28, 31). Those collating observational findings found inverse relationships between serum 25-hydroxyvitamin D levels and risk and severity of ARTIs (29) and risk of community-acquired pneumonia (30). Meta-analyses pooling evidence from RCTs focused on findings from vitamin D supplementation programs represent a higher level of evidence since they can establish a cause-and-effect relationship. The largest meta-analysis included data from 45 RCTs (n = 73,384 subjects) concluding that daily dosing regimens providing 400–1,000 IU (10–25 μg) of vitamin D were most effective at protecting against ARTIs (27). Earlier meta-analyses reported similar findings: that vitamin D supplementation lowered ARTI risk (28), particularly among those with profound 25-hydroxyvitamin D deficiency at baseline (28). Focusing on vitamin D supplementation, a separate meta-analysis (15 RCTs, n = 7053) observed a 6% risk reduction of clinical RTIs, but this was not statistically significant and heterogeneity among the included studies was high (I^2 = 57%) (31).

Evidence from individual RCTs has reported similar findings. Five studies reported that vitamin D supplementation reduced the incidence (32, 35, 37), duration and severity (36), and symptoms (34) of RTIs. Among asthmatic patients, Ramos-Martinez et al. observed that vitamin D reduced RTIs, an effect that correlated with higher sputum levels of IL-10, IFN-γ, and cathelicidin LL-37 (35). Vitamin D dosages used among the different studies were highly variable, ranging from just 10 IU (0.25 μg daily) (35) up to 4,000 IU (100 μg daily) (37). Similarly,

TABLE 1 | Summary of meta-analyses of vitamin D and RTIs with a focus on adults.

References	Details of studies included	Study type	Infection definition/form	Publication focus	Main findings
Jolliffe et al. (27)	45 RCTs ($n = 73,384$)	Meta-analysis [Update: additional RCTs added to Martineau et al. (28) meta-analysis]	ARTIs—The definition of ARI encompassed URI, LRI, and ARI of unclassified location (i.e., infection of the upper and/or lower respiratory tract). Form: Not Specified	RCTs of vitamin D supplementation	Protective effects against ARTIs were seen in trials where vitamin D was given: ○ Via a daily dosing regimen (OR 0.75, 95% CI 0.61–0.93) ○ at daily dose equivalents of 400–1,000 IU (OR 0.70, 95% CI 0.55–0.89) ○ for a duration of ≤12 months (OR 0.82, 95% CI 0.72–0.93)
Pham et al. (29)	14 studies of ARTI risk ($n = 78,127$) 10 studies for trend analysis ($n = 69,048$) 5 studies ARTI and vitamin D concentration ($n = 37,902$)	Systematic review and meta-analysis of observational studies	ARTI, defined as an acute infection of the respiratory tract in either the lower or upper airway or with the location not specified. ARTI was either self-reported or clinically confirmed. Form: Not Specified	Vitamin D status	Serum 25(OH)D concentrations were inversely associated with risk and severity of ARTI (pooled OR 1.83, 95% CI 1.42–2.37 and OR 2.46, 95% CI 1.65–3.66 comparing the lowest with the highest 25(OH)D category, respectively)
Zhou et al. (30)	8 studies ($n = 20,966$)	Meta-analysis of observational studies.	Pneumonia infection. Form: Not Specified	Vitamin D status	Community-acquired pneumonia patients with vitamin D deficiency [serum 25(OH)D levels <20 ng/ml] experienced a significantly increased risk of pneumonia (OR 1.64, 95% CI 1.00–2.67)
Martineau et al. (28)	25 eligible RCTs ($n = 11,321$; individuals aged 0 to 95 years)	Meta-analysis of RCTs	Classified as an upper respiratory tract infection, lower respiratory tract infection, and acute respiratory tract infection of unclassified location. Form: Not Specified	RCTs of vitamin D supplementation	Vitamin D supplementation lowered ARTI risk among all participants (OR 0.88 95% CI 0.81–0.96); effects were greater among those more deficient at baseline
Gysin et al. (31)	15 RCTs ($n = 7,053$)	Meta-analysis of RCTs	The first episode of clinical RTI was reported as cold/influenza-like illness and laboratory confirmed by standard microbiological methods. Form: Bacterial	RCTs of vitamin D3 supplementation	There was a 6% risk reduction of clinical RTIs with vitamin D3 supplementation, but this was not statistically significant (RR 0.94; 95% CI 0.88–1.00)

ARTIs, acute respiratory tract infection; CI, confidence interval; LRTI, lower respiratory tract infection; OR, odds ratio: RTI, respiratory tract infection; URTI, upper respiratory tract infection.

durations of RCTs were also wide-ranging, with the shortest being 4 weeks (34) and the longest, conducted in health care residents, being over a 12-month period (37).

Regarding pathological cause of infection, only four studies clearly specified whether these were bacterial (31, 35, 38) or viral (32). The remaining studies focused on the location, duration,

TABLE 2 | Summary of recent RCTs investigating the effect of vitamin D supplementation on RTIs in adults.

References	Study population	Study type	Intervention	Infection definition/form	Main findings
Arihiro et al. (32)	$n = 223$ patients with inflammatory bowel disease	6-month multicenter double-blind, placebo-controlled RCT	500 IU (12.5 μg) vitamin D or control daily	Influenza infection diagnosed using influenza virus test kits. Form: Viral	Incidence of URTI was significantly lower in the vitamin D group (RR 0.59; 95% CI, 0.35–0.98)
Slow et al. (33)	$n = 60$ vitamin D group, $n = 57$ placebo	6-week randomized, double-blind, placebo-controlled trial	Single high-dose vitamin D3 (200,000 IU)	Pneumonia that has been acquired outside of a hospital or health care setting. Form: Not specified	Vitamin D increased the complete resolution of pneumonia in participants with baseline vitamin D levels <25 nmol/L (OR 17.0, 95% CI 1.40–549.4), but this was of modest statistical significance ($p = 0.043$)
Jung et al. (34)	$n = 25$ male taekwondo athletes aged 19–22 years	4-week double-blind, placebo-controlled RCT	5,000 IU (125 μg) vitamin D3 or control daily	The Wisconsin Upper Respiratory Symptom Survey-11 (WURSS-11) was used. Form: Not specified	Serum 25(OH)D levels increased by 256% and were inversely associated with total URTI symptoms ($r = -0.435$, $p = 0.015$)
Ramos-Martinez et al. (35)	$n = 86$ patients with asthma aged 18–50 years	6-month double-blind, placebo-controlled RCT	10 IU (0.25 μg) calcitriol ($1,25\text{-}(OH)_2D_3$) or control daily	Respiratory infections in asthmatic patients. Form: Bacterial	Vitamin D supplementation reduced RTIs and reduced airways colonization by pathogenic bacteria
Shimizu et al. (36)	$n = 428$, aged 45–74 years	16-week double-blind, placebo-controlled RCT	400 IU (10 μg) vitamin D or control daily.	The Japanese version of Wisconsin Upper Respiratory Symptom Survey-21 (WURSS21) was used. Form: Not specified	Vitamin D reduces the duration of URTI, the physical severity of URTI, and the quality of life when suffering from URTI
Ginde et al. (37)	$n = 107$ longer-term care residents, aged over 60 years	12-month double-blind, parallel group, randomized controlled phase II trial	High dose (3,000–4,000 IU/75–100 μg day) or standard dose (400–1,000 IU/10–25 μg day).	Measured both upper (common colds, sinusitis, pharyngitis, otitis media) and lower (acute bronchitis, influenza, pneumonia) ARIs that required medical attention Form: Not specified	The high-dose group had 0.67 ARIs per person-year compared to 1.11 in the standard dose group (incidence rate ratio 0.60; 95% CI 0.38–0.94; $p = 0.02$)
He et al. (38)	$n = 39$ athletes during winter training	14-week placebo-controlled RCT	5,000 IU (125 μg) vitamin D or control daily	Measured changes in antimicrobial peptides. Form: Bacterial	Blood and salivary analyses showed that serum 25(OH)D levels increased by 130% and vitamin D increased SIgA and cathelicidin, which could improve resistance to respiratory infections

UPTI, upper respiratory tract infection; CI, confidence interval; OR odds ratio.

and/or severity of RTIs, but their source was not clearly defined or diagnosed.

DISCUSSION AND CONCLUSIONS

Presently, vitamin D guidelines in the United Kingdom have been set at 10 μg daily from October to March to keep bones, teeth, and muscles healthy (39). However, given updated meta-analytical evidence and a growing number of RCTs, combined with the global COVID-19 pandemic, it seems timely that this advice should be updated to encompass respiratory health with the required supplemental dose being re-evaluated. Clearly, vitamin D intakes should conform to recommended upper safety limits established by expert authorities with the European Food Safety Authority setting an upper limit of 100 μg/day for adults, including pregnant and lactating women (40).

Equally, supplementation should always be in addition to the consumption of a healthy, varied, and well-balanced diet. Nevertheless, a more desirable level of intake of vitamin D taking the latest evidence into account would be 2,000 IU (50 μg) daily to reduce the risk of ARTIs (41).

Regarding antibiotic use, more clinical studies evaluating the impact of vitamin D are needed as an outcome alongside ARTI incidence, symptoms, and severity. One Cochrane review evaluated evidence from seven studies where vitamin D was used as an adjunct to antibiotics to treat pneumonia, but findings were inconclusive (42). In Sweden, vitamin D3 supplementation [1,500–1,600 IU (37.5–40 μg) daily over 12 months] was found to significantly reduce antibiotic usage—from 20 to 15 days per person (43). Equally, future studies should clearly define the origin of pathological RTIs. It is possible that different vitamin D dosing regimens may be warranted for viral and bacterial infections, but there is presently not enough evidence to draw firm conclusions on this.

AMR poses a threat to future global health, and the current COVID-19 pandemic is highly damaging to health, societies, and economies. Urgent responses are needed. Supporting the immune system of the population in advance of exposure to infections (i.e., "immune prehabilitation") would reduce the number and severity of infections and reduce use of antibiotics. Vitamin D has multiple roles in supporting the immune system (21, 44, 45) and evidence from RCTs demonstrates that supplemental vitamin D reduces risk of acquiring RTIs (32, 35, 37) as well as their duration and severity (34, 36) and may reduce antibiotic use (43). There is also evidence that individuals with better vitamin D status are less likely to develop COVID-19 and severe COVID-19 (46–48). Given these observations, guidance of vitamin D intake should consider immune health, in addition to bone, tooth, and muscle health. Higher vitamin D intake in the population would reduce infections, result in infections being less severe and reduce use of antibiotics. The RCTs that form the current evidence base have highly variable designs including substantial differences in dose of vitamin D used, regularity of dosing, and duration of dosing. Thus, further clinical trials and meta-analytical approaches are warranted to clarify matters of dose, dosing regimen, and the precise relationship between vitamin D status and immune and respiratory health in different groups of the population including older people and different ethnicities.

AUTHOR CONTRIBUTIONS

ED and PC compiled, researched, wrote, and edited the review.

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Bronchiectasis—Could Immunonutrition Have a Role to Play in Future Management?

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Bronchiectasis is a chronic condition in which areas of the bronchial tubes become permanently widened predisposing the lungs to infection. Bronchiectasis is an age-associated disease with the highest prevalence in people older than 75 years. While the prevalence of bronchiectasis is higher in males, disease is more severe in females who have a poorer prognosis. The overall prevalence of the disease is thought to be rising. Its aetiology is multi-faceted, but a compromised immune system is now thought to play a central role in the pathology of this disease. Research has begun to study the role of malnutrition and certain nutrients—vitamin D and zinc—along with the role of the lung microbiome in relation to the management of bronchiectasis. Given this, the present mini review sets out to provide an overview of the state-of-the-art within the field, identify research gaps and pave the way for future developments and research investment within this field.

Keywords: bronchiectasis, lung health, respiratory tract infections, immunonutrition, inflammation

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INTRODUCTION

For a long time, bronchiectasis has been regarded as an "orphan disease," a disease so rare that it was not considered commercially viable to develop drugs to treat it (1). This led bronchiectasis to be regarded as unimportant and it was subsequently under-researched (2, 3). Now, into the twenty-first century, bronchiectasis is becoming an increasingly frequent prognosis and it has even been coined as an emerging global epidemic (4). In the United Kingdom (UK) there is evidence to suggest that both the prevalence and incidence of bronchiectasis are rising quickly (5). For example, for non-cystic fibrosis bronchiectasis, the UK incidence for women rose from 21.2 per 100,000 person-years in 2004 to 35.2 per 100,000 person-years in 2013 and for men from 18.2 per 100,000 person-years in 2004 to 26.9 per 100,000 person-years by 2013 (5). It was found that bronchiectasis is more common amongst those with higher socioeconomic status, although the reason for this is unclear (5). The British Lung Foundation's "*Respiratory Health of the Nation*" project estimated that ∼212,000 people in the UK are living with bronchiectasis – levels higher than previously thought, with the severity of the condition more prevalent in females, in those aged over 70 years and in the least deprived sectors of the population, which is in contrast to other respiratory disorders (6). Sex differences, with females portending to worse clinical outcomes has, in part, been explained by differences in sex steroid hormones which may affect airway microbiology (7).

Bronchiectasis is characteristically caused by abnormal dilation of the bronchi and a damaged epithelium which, in turn, impairs mucus clearance and facilitates bacterial infection (8, 9).

The condition is frequently accompanied by inflammation with symptoms manifesting as a chronic cough, sputum production and recurrent infections (10). For most people the causes behind bronchiectasis are "idiopathic" but some pathophysiological mechanisms include: dysregulation of the immune system, inflammatory conditions (e.g., bowel disease or arthritis), or structural lung problems (11). Amongst the array of causative factors behind bronchiectasis, the majority of these compromise the immune response in some form resulting in an impaired ability to fight infection (12).

Treatment strategies for bronchiectasis focus primarily on improving quality of life, preventing the frequency of flare ups and offsetting disease progression (8). Long term use of antibiotics—continuous or cyclical (during the winter months)—is typically recommended to stabilise the disease (13). Increasingly, there has been a growing body of evidence linking nutrition to immune function and protection against respiratory infections (14–16). Consequently, so-called immunonutrition could also have a role in bronchiectasis management – a field of science that appears to be underexplored. This mini-review considers the science linking nutrition to bronchiectasis management and describes how this could be further advanced in the future. The focus of the present review is on non-cystic fibrosis bronchiectasis.

IMMUNE DYSFUNCTION IN BRONCHIECTASIS

King et al. stated that "*the deficiency of the host immune response to bacterial infection is regarded as a primary requirement for the development of bronchiectasis*" (12). While a spectrum of causative factors underpin bronchiectasis, the majority of these compromise immune function and the ability to fight infection (12). Both the adaptive and innate immune responses are activated in bronchiectasis (12), with neutrophils having a central role in bronchiectasis pathology. Inflammation is driven by neutrophils that migrate to the airway in response to elevated concentrations of chemokines and pro-inflammatory cytokines, which are elevated in the airways of these patients (17). Neutrophils would typically phagocytose and kill pathogenic bacteria, but in patients with bronchiectasis these appear to have been disabled, possibly by the cleavage of phagocytic receptors by neutrophil elastase (17). An excessive release of neutrophil elastase is known to have damaging effects on the lungs, including impaired ciliary beat frequency, airway epithelia destruction, mucus gland stimulation and increased sputum production (18). Other mechanisms related to immune dysfunction are also evident in patients with bronchiectasis. For example, proteases produced in the lung as a result of the inflammatory response result in pathological dilation that is characteristic of bronchiectasis (12).

SPECIFIC IMMUNONUTRIENTS AND BRONCHIECTASIS

Optimal functioning of the immune system is central to human health, and nutrition is one of the main exogenous factors involved in the modulation of immune function (19). The British Thoracic Society presently provides guidelines for adults with bronchiectasis, which include advice on airway clearance techniques, use of mucoactive treatments, anti-inflammatory therapies, and antibiotic use (20). Nutritional advice is not presently included, perhaps because of an historic lack of relevant evidence. However, recent research has begun to investigate the role of nutrition in bronchiectasis and respiratory infection management.

It is now becoming relatively well-established that vitamin D supplementation could help to attenuate respiratory tract infections (RTIs) (21, 22). Vitamin D receptors are present in almost all cells of the immune system, vitamin D thus being important to the regulation of both innate and adaptive immunity (23). For example, vitamin D receptors are found on monocytes which differentiate into macrophages under the effects of $1,25(OH)_2D$ (24). Macrophages and dendritic cells also express their own 1α-hydroxylase isoenzyme which synthesises $1,25(OH)_2D$ intracellularly (25). Vitamin D receptors are further present on T-cells, B-cells and monocytes (26). As well as possessing immunomodulatory functions, vitamin D is also known to increase the antiviral responses of respiratory epithelial cells during infections (27). Vitamin D has been found to enhance neutrophil killing in patients with bacterial respiratory tract infections (RTIs) and to lower pro-inflammatory cytokine production induced by infected neutrophils (28). Amongst patients with cystic fibrosis, vitamin D status correlated with immunoglobulin concentrations and reduced CD279 programmed death 1 (PD-1) expression on $CD8^+$ T cells and the frequency of $CD8^+$ T cells co-expressing CD38 and human leucocyte antigen D-related (29). Vitamin D also induces antimicrobial peptide gene expression including that of cathelicidins and defensins which possess the ability to disrupt the integrity of the pathogens, resulting in their apoptosis (30). These mechanisms could, in part, explain some of the "antibiotic" actions of vitamin D (30). Together, these observations suggest that vitamin D could have a potential extended role to play in bronchiectasis management.

Clearly, the pathogenesis of bronchiectasis is multi-faceted with autoimmune disease, conditions of immune dysregulation, genetics, geography, and ethnicity having underpinning roles (9). As shown in **Table 1** several nutritional risk factors could also contribute to bronchiectasis pathology. These includes malnutrition, pro-inflammatory diets, suboptimal hydration status and vitamin D and zinc shortfalls. Malnutrition and an imbalance between energy supply and requirements can result in a negative balance between protein synthesis and breakdown, potentially affecting the prognosis of respiratory diseases (31). A growing body of evidence now links pro-inflammatory diets to disease-related fatigue whilst balanced diets comprised of whole-grains, polyphenols-rich vegetables and foods providing omega-3 fatty acids may attenuate fatigue related to chronic disease (33). Adequate hydration is important for mucociliary clearance, helping to reduce concentrations and cohesion (38).

Several trials suggesting a link between low vitamin D status and bronchiectasis have been published (**Table 2**). Scottish research measuring serum vitamin D levels in patients with bronchiectasis ($n = 402$) found that 50% were deficient, 43%

TABLE 1 | Bronchiectasis: potential modifiable nutritional risk factors.

Dietary component	Explanation and potential pathway(s)	Reference(s)
Malnutrition	Malnutrition (lack of adequate nutrition) can cause diverse alterations in the innate and adaptive immune responses. These include the involution of the thymus, reducing T cell numbers and responses and exacerbating the susceptibility to infections.	(31, 32)
Pro-inflammatory diets	Several foods and dietary patterns have been identified as pro-inflammatory, inducing secretion of inflammatory mediators, and free radicals. Those most studied include hydrogenated fats, processed foods, and refined sugars. In contrast, balanced diets comprised of whole-grains, polyphenol-rich vegetables and foods providing omega-3 fatty acids may attenuate inflammation (so reducing fatigue related to chronic disease).	(32–34)
Vitamin D	There is evidence from RCTs for protective effects of vitamin D on tuberculosis and likely evidence for protective effects on acute airway infection. As vitamin D deficiency is prevalent, daily oral vitamin D intake, e.g., 25 μg (1,000 IU) could be an inexpensive measure to ensure adequate status in at-risk groups. Vitamin D interacts with antigen presenting cells and T-lymphocytes to promote and to regulate different stages of immune response. It has also been found to modulate antimicrobial peptide pathways and induce immune activation in patients with cystic fibrosis.	(29, 30, 35, 36)
Zinc	Zinc is regarded as the gatekeeper of immune function and could be of benefit in instances when the immune system is functioning poorly. Zinc is thought to activate key signalling molecules and induce epigenetic modifications that underpin its roles as a gatekeeper of immune function.	(37)
Water	Rehydration could potentially help to reduce mucus concentration and cohesion. EFSA advise water intakes of 2 litres/day for females and 2.5 litres/day for males from food and beverage sources.	(38, 39)

TABLE 2 | Summary of studies of nutrients and the lung microbiota in bronchiectasis.

References	Type of study	Point of focus	Main findings
Niksarlioglu et al. (40)	Case-Control	Vitamin D	Vitamin D deficiency was common in patients with bronchiectasis and associated with significantly lower forced vital capacity.
Fagihi et al. (41)	Case Study	Vitamin D	Co-infection of SARS-CoV-2 and *Bordetella bronchiseptica* in a bronchiectasis case was treated with vitamin D supplementation (100,000 IU bolus dose and weekly dose of 25,000 IU), doxycycline and standard intensive care.
Woo et al. (42)	Observational	Lung microbiota	The lung microbiota of patients with non-cystic fibrosis bronchiectasis was relatively stable.
Bartley et al. (43)	Pilot Intervention Study	Vitamin D	Adults with bronchiectasis had higher than expected serum vitamin D levels and standard oral vitamin D3 supplementation further increased this and health-related quality of life measures.
Byun et al. (44)	Cross-sectional	Lung microbiota	Lung microbiota was no different between stable and exacerbated bronchiectasis.
Mirra et al. (45)	Pilot Cross-sectional study	Vitamin D	79% of patients with bronchiectasis had vitamin D deficiency-to-insufficiency; hypovitaminosis D was common.
Chalmers et al. (46)	Observational Study	Vitamin D	Vitamin-D deficiency was common in bronchiectasis and correlated with bacterial colonisation, inflammatory mediator levels in sputum and reductions in lung function.
Javadmoosavi et al. (47)	Cross-sectional study	Zinc	Serum zinc levels were lower in patients with bronchiectasis compared with controls.
Tunney et al. (48)	Cross-sectional	Lung microbiota	After treatment of patients with bronchiectasis for exacerbations (flare ups) no changes in lung microbiota composition were observed.

insufficient and only 7% had sufficient status (46). Additionally, 24% of the vitamin D deficient patients were colonised with *Pseudomonas aeruginosa*, had elevated sputum inflammatory marker levels and were more likely to have a decline in lung function at follow-up (46). A case-control study involving 130 Turkish cases with bronchiectasis observed that vitamin D deficiency was common and significantly associated with reduced forced vital lung capacity (40). Similarly, a pilot study conducted in New Zealand found that adults with bronchiectasis had a tendency towards sub-optimal serum vitamin D levels and weekly supplementation with 0.6 mg vitamin D3 (after a loading dose of 2.5 mg) significantly increased serum vitamin D levels (43). Amongst patients with primary ciliary dyskinesia (64% with bronchiectasis) hypovitaminosis D was evident in 79% (45). Interestingly, in a recent case study of a young male with severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2),

the virus which causes COVID-19, bronchiectasis and *Bordetella* bronchiseptica colonisation were treated successfully with a vitamin D3 intervention, doxycycline and intensive care (41).

Zinc is another nutrient known to support the function of many immune cell types and to benefit respiratory infections (49). Zinc is the chief regulator of signal transduction and cellular function, with zinc deficiency altering zinc homeostasis and molecular mechanisms including the actions of kinases, caspases, phosphatases, and phosphodiesterases which can exacerbate infection susceptibility (50). *In vivo* research also shows that zinc deficiency modulates the function and number of monocytes, neutrophils, natural killer, B-, and T-cells, with T-cell balance and function being particularly receptive to changes in zinc status (51).

To date only one study has investigated a potential role of zinc in bronchiectasis; the study found that serum zinc levels were significantly lower in patients with bronchiectasis compared with controls (47). The authors concluded that zinc could have extended benefits helping to modulate the immune system in these patients (47). Therefore, studies are now warranted that focus on zinc status/supplementation programmes in relation to specific markers of immune function (e.g., T-cell function), lung function and infection in bronchiectasis cases.

The microbiota also interacts with the local immune system. Most research has focussed on the role of the gut microbiota, which may affect both gastrointestinal and respiratory illness, but the lung microbiota is also now recognised to be important (52, 53). The lung airway microbiota is low in biomass compared to the gut, yet could be a powerful regulator of the immune system (54). As with the gut there is likely to be a bidirectional relationship between the lung microbiota and host epithelial and immune cells. For example, lung epithelial cells are being recognised as active effectors of microbial defence, contributing to both adaptive and innate immune function in the lower respiratory tract (55).

A small number of studies have begun to investigate the profile of the lung microbiota in patients with bronchiectasis (42, 44, 48). In general, the lung microbiota has been found to be relatively stable in these patients, with few differences between remission and flare-up of disease although comparisons with healthy adults are warranted (42, 44, 48).

DISCUSSION

Immunonutrition could be a means of reducing bronchiectasis-related infection and disease progression, which over time can result in impaired lung function. A number of nutrients support the immune system including vitamins B6, B9 (folate), B12, C, D and E, zinc, copper, iron and selenium and essential amino acids and fatty acids (15, 16, 56). There is now meta-analytical evidence available, which demonstrates that vitamin D supplementation reduces the risk of respiratory tract infections (21, 22, 57). In the UK, Biobank data shows that Asian and black residents have higher rates of vitamin D deficiency (54 and 35%) than Caucasians (12%) and socioeconomic deprivation, smoking and northerly latitudes increased the odds of vitamin D deficiency (58).

Zinc may also reduce risk of such infections and a recent rapid review of 118 publications concluded that zinc could reduce the risk, duration, and severity of SARS-CoV-2 and other respiratory virus infections through several mechanisms which include its potential to reduce inflammation, interfere with SARS-CoV-2 replication, improve mucocilliary clearance, promote antiviral immunity and reduce the risk of co-infection with pneumonia (59). Malabsorption syndromes and the consumption of high phytate-containing cereals in the developing world have been associated with high rates of zinc deficiencies (60). In addition to traditional essential nutrients, many plant-derived dietary components, including flavonoids, may also support the immune response and regulate inflammation. A meta-analysis of 14 studies reports that flavonoid supplementation reduced upper respiratory tract infection incidence by 33% compared with control, although differences between immune markers between groups were found to be modest (61).

As indicated earlier, there are observations of low vitamin D and zinc status being linked to bronchiectasis, but randomised controlled trials looking at effects on immune markers, lung function and infection with these two nutrients, and with other potential candidates, are lacking. This is an important research gap. Currently neither the British Thoracic Society nor the European Respiratory Society bronchiectasis guidelines include any nutritional advice (20, 62). Given the potential for nutrients to play a useful role in disease management, more research in this area is warranted. Current advice for the UK general population is to take a 10 microgram daily supplement of vitamin D to keep bones, teeth, and muscles healthy (63). However, intakes of 2,000 IU/day (50 micrograms/day) seem to be needed to reduce the risk of respiratory tract infections (16). Vitamin D deficiency may play a role in the pathogenesis of bronchiectasis and vitamin D supplementation could be a cost-effective therapeutic approach to help manage and attenuate the severity and progression of this disease (64), if it was supported by quality research evidence.

Regarding the roles of other nutrients and the lung microbiota and its modulation, there is presently not enough evidence to make any clear conclusions. Research on candidate nutrients other than vitamin D is warranted; high quality trials focusing on vitamin D, zinc, other nutrients, and flavonoids and immune- and bronchiectasis-markers would be worthy of future study (15, 16, 65). Research focusing on the gut and lung microbiota in patients with bronchiectasis over extended time periods and using larger sample sizes to capture disease heterogeneity during stability and at exacerbation is warranted (66).

CONCLUDING REMARKS

Bronchiectasis is a progressive immune-infective-inflammatory airway condition that results in a vicious cycle of repeated exacerbations and irreversible damage. It is becoming more common. Whilst conventional medical approaches are clearly

crucial to its management, immunonutrition could also have a viable role to play, with evidence for vitamin D currently being the most promising. Given growing interest in lung health, in part sparked by the SARS-CoV-2 pandemic, greater focus and investment in research is needed in this disease.

AUTHOR CONTRIBUTIONS

ED and PC compiled, researched, wrote, and edited the review. All authors contributed to the article and approved the submitted version.

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REVIEW
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Glycophosphopeptical AM3 Food Supplement: A Potential Adjuvant in the Treatment and Vaccination of SARS-CoV-2

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The world is currently experiencing the coronavirus disease 2019 (COVID-19) pandemic caused by Severe Acute Respiratory Syndrome-2 (SARS-CoV-2). Its global spread has resulted in millions of confirmed infections and deaths. While the global pandemic continues to grow, the availability of drugs to treat COVID-19 infections remains limited to supportive treatments. Moreover, the current speed of vaccination campaigns in many countries has been slow. Natural substrates with biological immunomodulatory activity, such as glucans, may represent an adjuvant therapeutic agent to treat SARS-CoV-2. AM3, a natural glycophosphopeptical, has previously been shown to effectively slow, with no side effects, the progression of infectious respiratory diseases by regulating effects on innate and adaptive immunity in experimental models. No clinical studies, however, exist on the use of AM3 in SARS-CoV-2 infected patients. This review aims to summarize the beneficial effects of AM3 on respiratory diseases, the inflammatory response, modulation of immune response, and attenuation of muscle. It will also discuss its potential effects as an immune system adjuvant for the treatment of COVID-19 infections and adjuvant for SARS-CoV-2 vaccination.

Keywords: COVID-19, AM3, glycophosphopeptical, food supplement, immunonutrition, cytokines, muscular damage, vaccination

INTRODUCTION

The world has experienced, and today continues experiencing, the COVID-19 pandemic caused by Severe Acute Respiratory Syndrome-2 (SARS-CoV-2). Its transmission probability has been estimated in the beginning of the pandemic in 1.4-2.5 (1) which has caused a rapid spread worldwide resulting in millions of deaths (2). SARS-CoV-2 infection causes a wide range of

symptoms. While a sizable number of patients present flu-like symptoms, others develop a severe condition associated with respiratory distress and pneumonia (3). These cases are also characterized by Acute Respiratory Distress Syndrome (ARDS), renal failure, septic shock or multi-organ failure, conditions that generally require hospitalization, intensive care unit (ICU) admission, and/or mechanical ventilation (4) and a multisystem inflammatory syndrome (5). In relation to the spectrum of the COVID-19, 81% suffer from a mild illness, 14% require hospitalization, of which 6% suffer from serious illness, and 5% need admission to hospital and/or ventilation in the ICU. Although the case fatality rate varies by country, overall it is roughly 2% (6).

The current availability of drugs to treat COVID-19 infections remains limited to supportive treatments. These are the main methods of care such as supplemental oxygen and mechanical ventilator support in severe and critical cases (7). However, drugs such as antiparasitics, including antimalarial drugs based on *in vitro* or animal-model antiviral activity, antibiotics, broad-spectrum antivirals and other modern therapeutic agents have been reused (8). Clinical trials have focused on the antimalarial drugs chloroquine and hydroxychloroquine, the antibiotic drug azithromycin, and convalescent plasma transfusion (9). The knowledge of SARS-CoV-2 virology provides a considerable number of possible targets for antiviral drugs. However, there are still no conclusive data on the efficacy of antivirals such as Ribavirin, Oseltamivir, Favipiravir, and the anti-tumor drug Plitidepsin. Their substantial toxicity suggests that it has limited value for the treatment of COVID-19 (10). None of these treatments have been approved by any regulatory body yet. Still, Remdesivir is currently the most promising as it has been granted an Emergency Use Authorization by the United States Food and Drug Administration (FDA or USFDA) as it undergoes clinical trials Phase III. Remdesivir reduces the stay in intensive care, although without significant clinical effects (11). Another option is the use of several antivirals in combination (Lopinavir, Ritonavir, Ribavirin) associated with Interferon 1β which has been shown to reduce symptoms and reduce the temporality of the infective phases of COVID-19. However, the therapeutic regimen is complex and its availability in hospitals is limited (12).

Therefore, the temporal need to find an effective antiviral agent is critical, and except for Dexamethasone, has not been shown to be any effective treatment. Dexamethasone, a glucocorticosteroid, is able to reduce the body's immune response which could modulate the COVID-19 multisystem inflammatory syndrome (13). This drug offers the most cost-effective treatment for COVID-19 which can reduce mortality by 20% in patients on oxygen therapy and by 30% in patients on automatic ventilation (14). It also has an anti-viral effect by binding to the hormone cortisol, stimulating the production of anti-inflammatory cytokines and inhibiting the secretion of pro-inflammatory cytokines that cause COVID-19 pneumonia (15).

An alternative approach to prevent COVID-19 infections is vaccination. This strategy represents the most effective prevention measure to help end the pandemic (16). More than 100 prototype vaccines against SARS-CoV-2 have been tested, and a limited number have been approved by the regulatory authorities. Briefly, COVID-19 vaccines aim to expose the body to an antigen that does not cause disease but, rather, sparks an immune response that blocks or destroys the virus in the face of an attempted infection (17). Such strategies can be categorized into: viral vaccines, attenuated viruses or inactivated viruses; vaccines containing the genetic instructions of a protein, nucleic acids in the form of DNA or RNA; vaccines with viral vectors, replicative vectors (attenuated measles) or non-replicative vectors adenovirus); and vaccines of a protein nature, by injecting subunits of COVID-19 structural proteins or viral structural particles that mimic the structure of SARS-CoV-2 (18). Currently, the process of vaccination against SARS-CoV-2 is slow among the majority of the countries, having vaccinated only 10% of the world's population (19). As such, new modalities are needed to reduce the burden of SARS-CoV-2.

In this context, alleviation of symptoms or enhancement of the healing process by other bioactive molecules with health-promoting properties (e.g., essential nutrients, herbal extracts, herbal extracts, phytochemicals and nutraceuticals) may offer an alternative strategy when no effective pharmacological treatments are available (20). Another alternative may be the use of natural substrates, such as the glucans, a group of substrates with biological immunomodulatory activity (21). These immunomodulators are molecules that have been previously used in clinical medicine and have been shown to improve health outcomes (22).

We describe in this paper a hypothetical therapeutic option of the biological effects of glucans and, more specifically, of the natural glucan glycophosphopeptical AM3 against COVID-19. Based on previous research that has documented positive effects on respiratory diseases (23, 24), regulation on inflammatory response (25–27), modulation of immune response (25–29), attenuation of muscle (29–31) and lung damage (32). This manuscript discusses AM3 as a potential adjuvant therapeutic agent, which could play a role in the prophylaxis or amelioration of symptoms associated with COVID-19. We also describe the potential benefits of AM3 as an immune system adjuvant for the control of SARS-CoV-2 infections through immunoprophylaxis, based on previous studies (33, 34).

GLYCOPHOSPHOPEPTICAL AM3

Any foreign antigen such as an infectious agent stimulates the immune system to some degree. Such heightened activity is accompanied by an increased rate of metabolism. As a result, the body needs some substrates to induce the production of mediators and effectors of the immune response. Therefore, additional energy sources, which are substrates and molecules, which may come from dietary intake, are required for biosynthesis of immune mediators for optimal functioning of the immune system (35, 36). Some of these substrates are glucans, molecules that perform preliminary innate actions triggered by pathogen-associated molecules (37). Glucans are linear glucose homo-polysaccharides linked to β-(1→3) and

β-(1→4). Differences in the linkages and branching influence the size of the molecule, its tertiary structure, electrical charge, conformation in solution (triple or single helix, or random spiral), and its solubility properties (38).

AM3 is a glycophosphopeptide composed of Candida utilis yeast phosphorylated glucomannan polysaccharide and Ricinus communis protein in a 5: 1 ratio (polysaccharide: protein). AM3 has been commercialized as an oral pharmaceutical product marketed as Immunoferon® by Cantabria-Labs (Santander, Spain). The Spanish Agency for Food Safety and Nutrition (AESAN) categorizes AM3 as "Food Supplement". The polysaccharide element of AM3 is a phosphoglucomannan-type B-glucan (GLPH-1; approximately 15 kDa), which contains repeating polysaccharides linear chains (10-40 repeats), with β(1→6) and β(1→2) links between and within the mannose and glucose residues in a ratio of 12: 1 (mannose: glucose). The protein element is a member of the 2S albumin family of proteins derived from RicC3 (27, 39). The protein fraction of RicC3 (12.0 kDa) consists of two subunits of different sizes that form a heterodimeric structure with very stable disulfide bridge bonds. Moreover, RicC3 has a five-helix bundle folded into a right-handed superhelix (40). In short, in AM3 the active ingredient is a 5:1 (w/w) mixture of polysaccharide and protein (AM3) (27).

The biopharmaceutical properties of AM3 allow it to produce an immunomodulatory action. AM3 is characterized by a protein/polysaccharide fraction that confers high resistance against enzymatic degradation and acidic pH in the stomach (27, 40). The potential beneficial effect of AM3 depends on its ability to reach its targets. However, once in the body AM3 must pass through a series of physiological barriers that can override it. As such, digestive enzymes and/or blood proteases can hydrolize and inactive AM3. However, the disulfide bridges of RicC3 allow AM3 to be a very stable compound, resistant to denaturation, and scarcely modifiable to proteolytic cleavage. This allows its high bioavailability. Furthermore, AM3 is not altered by liver metabolism and does not affect the hepatic bioconjugation system. Therefore, it does not alter the effect of drugs co-administered with AM3 (29, 41, 42). In this way the polysaccharide/protein structure of AM3 remains virtually unchanged and confers high bioavailability. This situation allows high concentrations of AM3 in the bloodstream to be achieved and to generate inactive fragments (29, 41, 42).

The polysaccharide fraction of AM3 interacts with endogenous mediators associated with intestinal lymphatic tissue. After AM3 polysaccharide has been absorbed and entered into the blood system, it interacts with the circulating dendritic cells (DCs) (37, 43, 44). These immunomodulatory properties are useful in the clinical setting in reducing decompensations and exacerbations in chronic obstructive pulmonary disease (COPD) patients caused by infectious respiratory diseases (viral and bacterial) (24, 45) with no side effects (25–28, 31, 33, 43, 44, 46–49). Thus, AM3 in COPD patients stimulates recovery of T-cell proliferation, restores IFN-γ production, increases the number of natural killer cells (NKC), and restores phagocytic activities. As such, a decrease in the rate of COPD exacerbation was observed in patients (24, 45). Additionally, AM3 was able to spark non-specific immune-system modulation (increase in antibody-producing B lymphocytes) in mice (47) without any alteration of toxicity indicators such as lactate dehydrogenase (LDH) and glutamic oxaloacetic transaminase (GOT) levels. Precautions, however, should be taken in pregnant and hypercalcemic patients (29, 31). Due to the effectiveness of AM3, a prophylactic setting is proposed in certain respiratory infections (like COPD exacerbations without significant toxicity. Therefore, AM3 may have potential use as adjuvant therapy for COVID-19 diseases by way of immune modulation (**Figure 1**).

ROLE OF AM3 IN THE SARS-COV-2 IMMUNE RESPONSE

The immune response against viruses consists of two phases. The first is non-specific and starts immediately after the entry of the virus into the host; this is called the innate immune response. The second is far more specific and may take some time to appear this is called the called adaptive or acquired immune response. Overall, the immune response plays a fundamental role in SARS-CoV-2 infection. It is necessary, therefore, to overcome and eliminate the virus but also appears to be responsible for the onset of severe and life-threatening disease. The severe damage to lung tissue that sometimes results from COVID-19 primarily reflects inflammation caused by an exaggerated immune response against the SARS-CoV-2 (50, 51).

Impact on Innate Immunity

AM3 and the Production of Natural Killer Cells

Natural killer cells (NKCs) act *via* extracellular death receptors and by exocytotic release of their content in the form of cytolytic granules. NKCs can eliminate virus-infected cells (44), restrict viral replication (52), and contribute to the early immune responses to viruses (53). Some NKC-activity abnormalities exist among activity in patients with common human respiratory viruses such as influenza virus, respiratory syncytial virus (RSV), probably because they have evaded NKC responses (54).

NKCs have been associated with cytokines such as interferon (IFN), interleukin-2 (IL-2) and interleukin-12 (IL-12) (55). IFN activity allows recruitment of pre-NKCs and enhancement of the cytolytic ability of active NK cells. Further, IL-2 and IL-12 maintain some spontaneous NK cell activity (46, 56). In animal models, AM3 (30 mg/kg/day or 60 mg/kg/day) significantly increased cytotoxic activity of NKCs through a positive induction of IFN and IL-2 (46). In this sense, Rojo et al. (57) administered AM3 (30 mg/kg/day) to several groups of mice 2, 3 or 7 days/week. Each *in vivo* treatment of AM3 improved the immune system's effector functions by enhancing IL-2 and NKC functions, after 15 days of treatment. Moreover, in mouse models AM3 (150 mg/kg/day) this effect lasted for 4 consecutive days, induced IL-12, IFN production, and boosted NKC, immune responses (58).

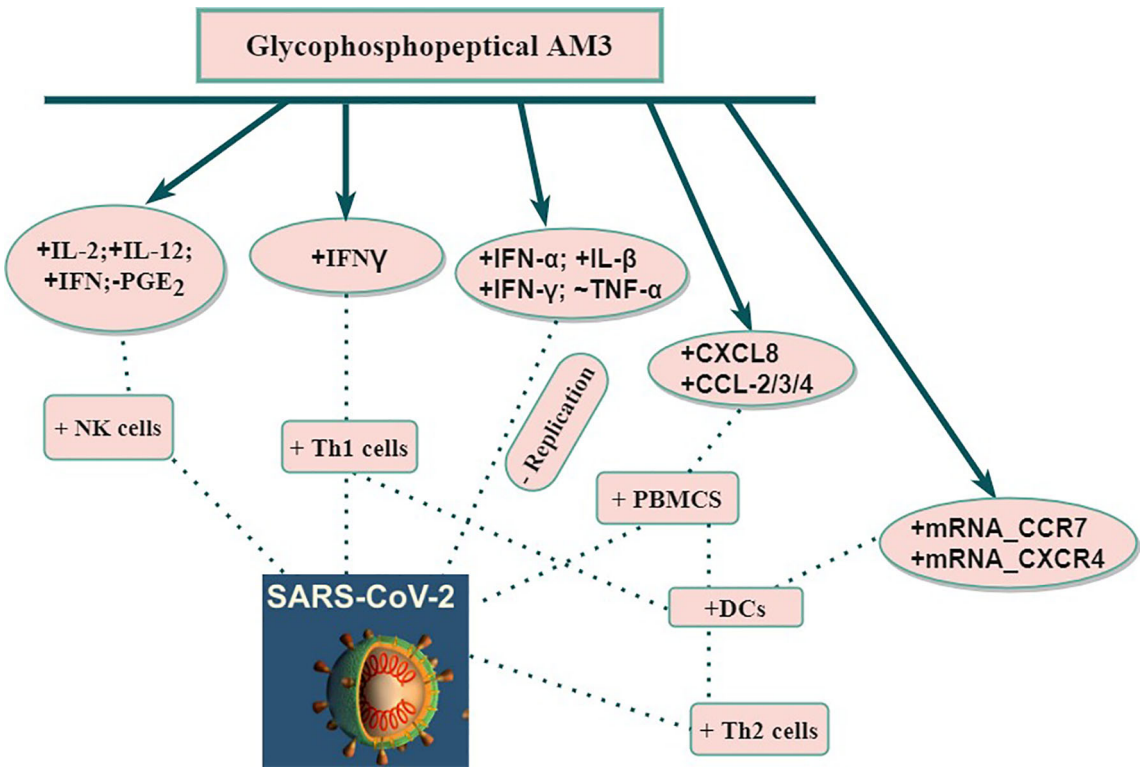

FIGURE 1 | Potential of use AM3 on immune response against SARS-CoV-2. CCL, Chemokine (C-X-C motif); CCR, Chemokine (C-C motif) Receptor; CXCL, Chemokine (C-X-C motif) Ligand; CXCR, Chemokine (C-X-C motif) Receptor; DCs, Dendritic cells; IFN, Interferon; IL, Interleukin; mRNA, Messenger RNA; NK, Natural Killer; PBMCS, Peripheral blood mononuclear cells; PGE, Prostaglandin; SARS-CoV-2, Severe Acute Respiratory Syndrome-2; Th1, T helper 1; Th2, T helper 2; TNF, Tumor Necrosis Factor; +, Stimulation; -, Inhibition; ~, Modulation.

On the other hand, AM3 inhibits prostaglandin-synthesizing cells directly or indirectly through an inhibition of the intracellular cyclic adenosine monophosphate (cAMP) (46). Prostaglandins (PGs), especially PGE2, are involved in the efficacious activity of NKCs (59, 60). Although the mechanism is unknown, AM3 probably inhibits the generation of PGE2 and/or blocks the E-type prostanoid receptor 4 (EP4). The activation of the EP4 receptor is necessary for the suppression of NKC function (61).

AM3 supplementation has been applied for diseases of the respiratory system. The clinical effect of AM3 treatment in chronic bronchitis, in which NKC depression occurs, is improving physiological NKC levels (23). NKC cytotoxicity peaked at 2 days after AM3 treatment; it remained elevated above control values for up to 8 days after a regimen of 3g/day of oral supplementation over 60 consecutive days in mice (58). Moreover, in patients with COPD, AM3 was able to stabilize diminished NKC function (24). In one study (24), 60 COPD patients received AM3 during 90 consecutive days at oral doses of 3g/day. The results showed that clear defects in the immunity of COPD patients are counteracted by stimulation of peripheral blood cells, especially polymorphonuclear neutrophils (PMNs), NKC, and monocytes/macrophages (MM) by way of AM3 supplementation (24).

In the case of COVID-19 infections, Demaria et al. (62), have observed a dysfunctional state combined with a decrease in NKCs in the blood and lungs of COVID-19 patients, which suggests that NKCs do not participate in the hyper-inflammatory characteristics of ARDS. Hence, AM3 supplementation may stimulate cytotoxic NKC in COVID-19 patients. This is primarily due to NKC's crucial role in antiviral immune responses and their contribution to the early immune responses to viruses (53). This could attribute some therapeutic properties to AM3 in COVID-19 patients.

AM3 and Cytokines

The use of AM3 has been demonstrated to be effective in modulating cytokines (25, 26, 43, 44). Supplementation of AM3 has been correlated with a significant increase in the production of interleukin-10 (IL-10) and IL-12 cytokines by DCs (43, 44). These results are similar to those described by Lagenkamp et al. (63) in stimulating DCs with LPS. IL-12 is essential as a performance link between innate and adaptive immunity. In addition, IL-12 stimulates the production of IFN-γ in NK and T cells. Finally, IL-12 drives/boost an immune response by means of Type 1 T helper cells (Th1) (64).

IL-10 is a cytokine that develops a primary immune response based on Type 2 T helper cells (Th2). IL-10 also has

anti-inflammatory and immunomodulatory properties. Thus, IL-10 is secreted by DCs, T cells, and macrophages (65). IL-10 plays an important role in limiting IL-12 production and downregulation of the inflammatory response (66). Finally, Martín-Vilchez et al. (43) have described that the use of AM3 may stimulate IFN-γ production, which is a Th1-stimulating cytokine, without increasing interleukin-4 (IL-4), which polarizes the immune response with Th2.

IL-4 triggers eosinophilia that accompanies airway inflammation. AM3 generally promotes an increase in IL-12, IFN-γ associated with unaffected IL-4 and stimulates a T-lymphocyte response through Th1 (43, 62). Such results have suggested the application of AM3 as an immunological enhancer of the host response against viral infection and novel adjuvant anti-viral treatment.

COVID-19 has established that the hyperinflammatory response induced by SARS-CoV-2, which contributes to the pathogenesis, is a major cause of disease severity and death in infected patients (67). In the early stages of the infection, a group of cytokines and pro-inflammatory chemokines are expressed including interleukin-1β (IL-1β), IL-2, IL-6, interleukin-8 (IL-8), both IFN-α/β, tumor necrosis factor (TNFα), CCL, CCL3, CCL5, CCL2, and IP-10. In the hyperinflammatory phase, patients with severe COVID-19 exhibit higher levels of IL-2, IL-6, IL-7, IL-10, IP-10, MCP1, TNF-α, macrophage inflammatory protein 1 alpha (MIP1A), and granulocyte-colony stimulating factor (G-CSF) than patients with moderate infections (early inflammatory phase). The fluctuations of these cytokines IL-6, and TNF-α exceed the physiological range (68). Thus, the overproduction of these cytokines and chemokines may contribute to lung damage and potentially fatal respiratory complications. This cytokine storm probably down-regulates innate and adaptive immunity against SARS-CoV-2 infection (69, 70).

There is a relationship between the immune system and the hypothalamus-pituitary-adrenal (HPA) system manifested by the secretion of glucocorticoids and other HPA-derived molecules. In COVID-19, the inflammatory response stimulates an initial acute phase characterized by monocyte/macrophage activation and the expression of pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 (49, 71). This triggers fever and the production of acute phase proteins of hepatic origin with a potent anti-protease activity that allows attenuation of inflammation and tissue destruction (28).

Brieva et al. (28) have reported that doses of 3, 6 and 9 mg/kg of AM3, dissolved in water for oral administration, stimulated anti-protease activity in hepatocyte cultures. AM3 has a plausible anti-inflammatory effect because it inhibits TNF-α and IL-6 induction by LPS in murine models (27, 28).

In athletes, strenuous exercise (elite competitive sport) has been associated with changes in cytokine production, specifically with increases in plasma concentrations of IL-1, IL-6 and TNF-α to supra-physiological levels (3, 72). The functional and potentially clinical significance of competitive sport-induced alterations in inflammatory cytokines results in generalized inflammation of the body, tissue damage, myalgia, alteration of the immune system (susceptibility to infection), fever, and chronic fatigue (73). These situations somehow coincide with the situations described in COIVD-19 patients. Thus, it is necessary to regulate the immune system as an essential therapeutic objective in inflammatory states such as strenuous exercise and COVID-19 infections. For example, researchers have observed that in elite cyclists, if treated with AM3 at a dose of 3 g/day for periods of 65 and 180 consecutive days, IL-6 and TNF-α were significantly reduced (25). Moreover, a significant increase in TNF-α receptors were observed with respect to the untreated group of AM3 (25, 26). TNF-α receptors have anti-inflammatory properties because they allow binding and neutralizing circulating and membrane-bound TNF-α (26). These immunomodulatory effects are a consequence of the oral administration of AM3 (25–29) and could be applied as a potential therapeutic adjuvant for SARS-CoV-2 treatment.

Alternatively, AM3 increased the plasma levels of corticosterone, which is also stimulated by HPA during the inflammatory process (27). Corticosterone and other glucocorticoids have been described to modulate the expression of mRNA of TNF-α (74). This supports the idea that the AM3 pathway is through direct interaction with HPA to control plasma TNF-α elevation during inflammatory processes (28). The potential modulation of inflammation of AM3 (**Figure 2**) causes a reduction of IL-6 and its ability to neutralize soluble TNF-α or to block TNF receptors from binding to their ligands. These multiple biological activities may attenuate the cytokine storm syndrome which often contribute to the severe pathogenesis of COVID-19.

An alternative to TNF-α hyper-response modulation in COVID-19 infections is the use of anti-TNF-α antibodies (75). However, most drugs that target TNF-α also block bioavailable TNF-α that monocytes and T cells produce, increasing the susceptibility to viral or bacterial coinfection or reinfection (76). AM3 normalizes the TNF-α production (27); in particular, AM3 inhibited TNF-α production by 90% compared to sera from placebo-treated mice. Taken together, these data suggest a regulatory role of AM3 in the response to the increased level of TNF-a production to physiological levels. Although many AM3 studies have demonstrated a high safety profile, its use must be monitored in COVID-19 infections.

On the other hand, the most relevant anti-viral defense element of innate immunity is the action of IFNs to limit and fight against viral infections. IFNs recruit neutrophils, control infection, modulate inflammation and develop the initial containment against invasion by COVID-19 (77, 78). IFN provides a response that would block the spread of the virus and allow the body the time necessary for the generation of a more specific and potent immune response (79, 80).

AM3 is also an immunological response modifier of IFN (46). Moya et al. (46) have observed increases in serum IFN concentrations when AM3 and IFN inducers (Newcastle disease virus (NDV) and/or bacterial lipopolysaccharide (LPS)) were coadministered in BALB/c mice (45). However, investigators observed that AM3 is not an IFN inducer per se. The IFN's

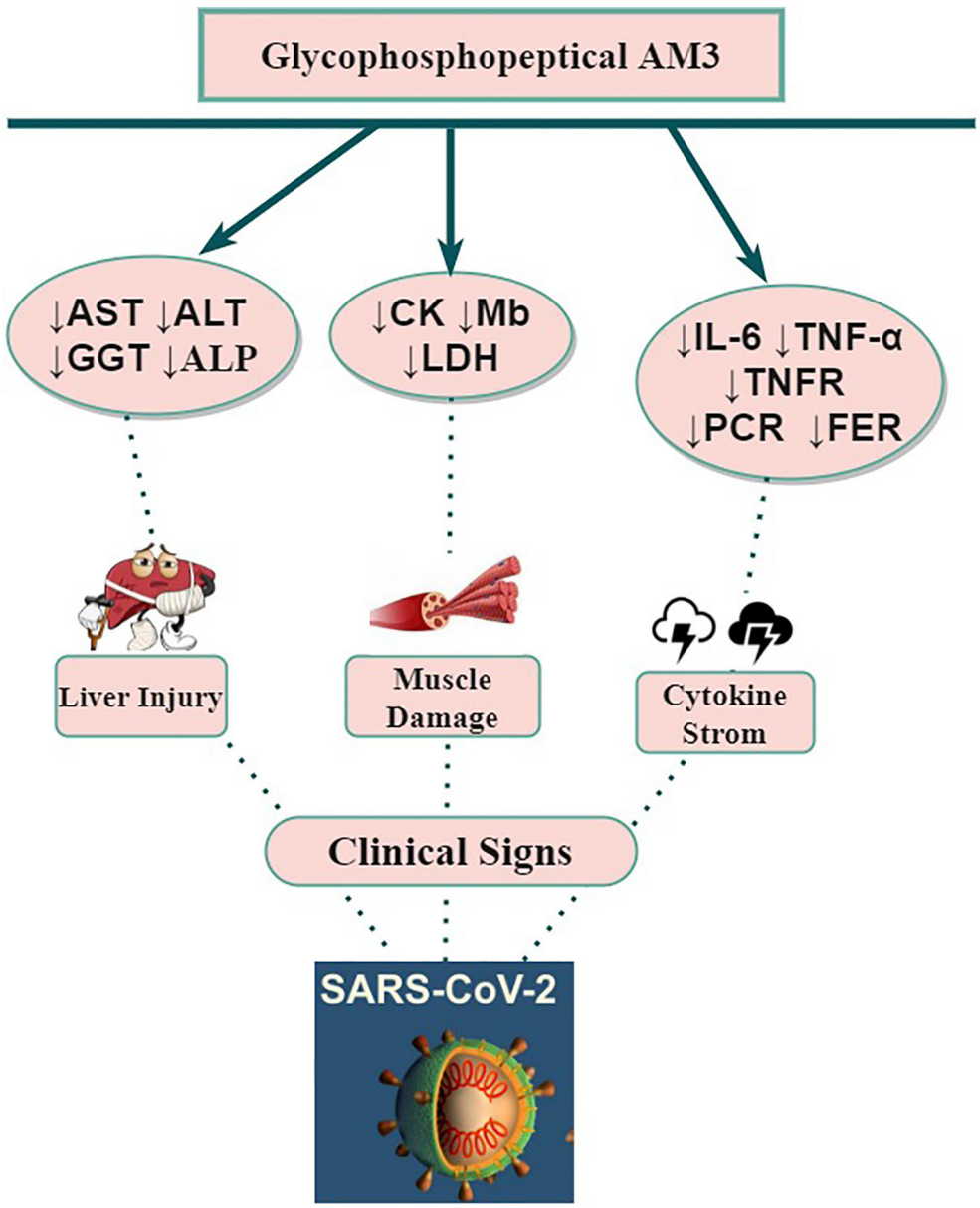

FIGURE 2 | Potential beneficial impact(s) of AM3 supplementation on COVID-19 clinical features and outcomes. ALP, Alkaline Phosphatase; ALT, Aspartate Alanine aminotransferase; AST, Aspartate Aminotransferase; CK, Creatine Kinase; Gamma Glutamyl Transpeptidase; IL, Interleukin; LDH, Lactate Dehydrogenase; Mb, Myoglobin; SARS-CoV-2, Severe Acute Respiratory Syndrome-2; TNF, Tumor Necrosis Factor; TNFR, Tumor Necrosis Factor Receptor; ↓, Decrease.

inhibitory effect reflects impaired NDV binding to cell-surface receptors because of viral glycation inhibition that blocks early interaction between the virus and host cells (81). Although, the type of IFN is not clearly specified, the results showed that in early stages AM3 act as a stimulator of NK cytotoxicity through IFN (46). In this sense, Type I IFNs act directly on NK cells to promote their activation, cell cycle entry and cytotoxic function in early stages viral infection (82). This might suggest that the IFN involved was IFN-I (α/β). Moreover, AM3 promotes natural immunity that is related to the induction of endogenous IFN-γ production in animal models when given at doses of 150 mg/kg/day for 4 consecutive days and patients with chronic bronchitis (3g/day during 60 consecutive days) (58). As described above, AM3 could exert different effects on each type of IFNs.

Host cells detect SARS-CoV-2 ribonucleic acid (RNA) in the cell cytosol and activate the IFN synthesis pathway. IFN secretion protein synthesis in infected host cells to prevent the virus from using "cell factories" to produce its proteins and replicate. This IFN activity causes cell death, which enters a process called apoptosis (83).

However, patients with severe COVID-19 infections have a lower expression of IFN-γ related to the decrease and impairment of cluster of differentiation 4+ (CD4+), cluster of differentiation 8 + (CD8+), and NKC (84). A high analytical interleukin-6 (IL-6)/ IFN ratio seems to predict severe COVID-19 disease and lung damage due to the cytokine storm (85). In this way, IFN-I levels are decreased (absence of IFN-β; low IFN-α production) which is a clinical feature of severe COVID-19 (81). SARS-CoV structural proteins, envelope proteins (EP), membrane proteins (MP), nucleocapsid phosphoproteins (NP) (86), and in SARS-CoV-2 two non-structural polyprotein (open reading frame [ORF] ORF1a and ORF1b) inhibit the release and secretion of IFN-I (87, 88). The viral proteins block IFN-I signaling by the Janus kinase (JAK) pathway, and consequently, decrease the signal transducer and the activator of signal transducer and activator of transcription protein (STAT1) (86). To solve this situation, the use of AM3 may effectively increase the levels of IFN (46, 58). IFN acts on neighboring cells and there, it activates genes that confer resistance to the infection trough antiviral and immunomodulatory activity which could attribute some therapeutic properties to AM3 against SARS-CoV-2 (83).

Interaction Between AM3 Supplementation and Nitic Oxide Production

Nitric oxide (NO) regulates physiological functions in the cardiovascular, nervous, muscular, and immune systems. In the immune system its action is non-specific towards tumor cells, virus or microorganisms, but it has also been associated with mechanisms of tissue damage, as well as in the inhibition of lipid oxidation by the lipoxygenase (LOX) and cyclooxygenase (COX) pathway (89). NO impairs the dissemination of immune cells and inflammatory points by altering cell adhesion and activation (90).

The synthesis of NO through one of the nitric oxide synthase isoforms, inducible Nitric Oxide Synthase (iNOS or NOS-II), is located in the smooth muscle cells and in the monocytes and macrophages. Expression of iNOS occurs in association with the local and/or systemic inflammatory response (91). iNOS is associated with inflammatory diseases of the airways and in the body's defense against infection. The iNOs generate more than 1,000 times the NO than the other nitric oxide synthase isoforms (eNOS and cNOS) and their production continues for a long period (92). As in the case of COVID-19 patients, their effect can be much more generalized, which can constitute a pathogenic mechanism *via* amplification of the inflammatory response with potentially harmful consequences. NO can enhance the inflammatory response by readily binding to the superoxide anion generating peroxynitrite ions that act directly on inflammatory cells. Elevated concentrations of NO can produce vascular damage in the endothelium causing toxic shock (93). This is similar to other inflammatory conditions, such as rheumatoid arthritis, chronic inflammatory bowel disease and atherosclerosis where excessive NO production by iNOS exacerbates tissue damage (94).

Lung injury caused by COVID-19 often evolves rapidly with ARDS followed by multiple organ failure due to a cytokine storm (79). In this way, bronchial epithelial cells produce NO, associated with overexpression of iNOs, after exposure to pro-inflammatory cytokines such as IL-1β and TNF-α (95). IL-1β and TNF-α will enhance the effects of NO. This process is like other chronic respiratory diseases such as asthma and COPD with a high inflammatory component (96). Dexamethasone is used in COVID-19 patients undergoing invasive mechanical ventilation and/or supplemental oxygen to reduce mortality (97). Dexamethasone acts by inhibiting the nuclear factor κβ (NF-κβ) of epithelial cells which decreases NO production (90). Corticosteroids can significantly benefit pulmonary function in ARDS patients, but they can also have adverse effects, which may have a detrimental impact on long-term outcome (98). AM3 supplementation has no known side effects that compromise other functions of the body that would compromise health (25–28, 31, 33, 43, 44, 46–49) and modulate NO production in mice (32). Specifically, the expression of iNOS inhibition resulted in a significant decrease in serum NO levels after treatment with AM3 (3 mg*Kg) for 6 consecutive days (32). Altogether, these results suggest that AM3 modulates the NO response as well as its possible role in the control of the inflammatory response associated with IL-6 and TNF-α (25, 32). Thus, AM3 could provide an adjuvant therapeutic option in order to reduce levels of dexamethasone doses and its side effects.

Impact on Adaptative Immunity

AM3 Effects on Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMCs) are critical components of the immune system (99). PBMCs include various types of lymphocytes (T cells, B cells, and NKCs), DCs, and monocytes (99).

AM3 and AM5 (active product molecule of AM3) act on PBMCs by stimulating the immune response (48, 49). It inhibits hepatitis B virus (HBV) replication, deoxyribonucleic acid (DNA) synthesis, and viral antigen expression through activation of PBMCs (49). Furthermore, AM3 stimulates secretion of IFN-α, IFN-γ, IL-β and modulate TNF-α (49), which have properties that indirectly control viral infections by PBMCs (71, 74). The SARS-CoV can infect and replicate in peripheral blood mononuclear cells (PBMCs) (100), invasion by SARS-CoV-2 could trigger deregulation of humans PBMCs. In this sense, *in vitro* SARS-CoV-2 infection of human PBMCs revealed they are susceptible cell types (69). Although the precise mechanism of cell invasion is unknown, investigation of apoptotic markers on T lymphocytes and CD147 expression levels could explain the mechanism of invasion and/or replication of SARS-CoV-2 in PBMCs.

Another mechanism that allows indirect AM3-control of viral infections is the activation of human DCs derived from human monocyte-derived dendritic cells (MDDCs) (44). MDDCs have been shown to initiate and maintain responses to pneumonia and lung inflammation, often playing a role in resolution. Their interaction with COVID-19 remains unclear but could be effective (67). MDDCs include the main complex of major histocompatibility (MHC) classes I and II, as well as molecules for co-stimulation (101). AM3 has a potential similar to LPS in

the expression of co-stimulatory molecules and MCH in patients infected with HBV that control chronic HBV infection (43).

Although there is no evidence yet of the potential of AM3 against COVID-19, the boost of the immune system *via* PBMCs or MDDCs may help to develop an antiviral immune response in these patients as adjuvant therapeutic approach. Because these immune cell subsets are altered during severe clinical stages of COVID-19 (102). Additionally, with increasing disease severity monocytes and MDDCs are substantially altered in number and function in blood and lungs during COVID- 19 infections (102). In severe and critical stages of COVID-19 infections, levels of neutrophils, lymphocytes (80% of patients present lymphopenia with a significantly alarming decrease between 80-100% of CD4+ and CD8+), and DCs in peripheral blood are reduced (103, 104).

DCs are the principal antigen-presenting cells for T cells involved in the innate and adaptive immune system. They recognize pathogen-related structures and stimulate T-cell activity (105). Dendritic cell-specific intracellular adhesion molecules (ICAM) - dendritic cell-specific ICAM-grabbing non-integrin (DC-SIGN) is a DC-specific adhesion receptor that binds with high affinity to ICAM3 that recognizes high mannose glycan that are present on pathogens (106). DCs are abundant in the lung and have a maximum peak of activity in early and severe stages of COVID-19 infections (79).

DCs infected with SARS-CoV-2 suggest the possible exacerbation of immunopathology. Nonetheless, the possibility of increased recruitment, accelerated maturation, and activation of DCs could combat COVID-19 infection (107, 108). AM3 upregulates C-X-C Motif Chemokine Ligand 8 (CXCL8), C-C Motif Chemokine Ligand (CCMCL), CCL2, CCL3 and CCL4 that are implicated in the conscription and maturation of DCs. DCs must migrate to the lymph nodes, where they will present antigens to the T-lymphocytes to perform their immune function (109). This process depends on the expression of the cc-chemokine receptors 4 (CCR4) and C-X-C chemokine receptor type 7 (CXCR7). CCR7 also regulates migration speed, survival, and differentiation in the CDs (110). Moreover, AM3 and LPS upregulate expressions on messenger RNA (mRNA) for the chemokine receptors CXCR4 and CCR7. Consequently, AM3 might be useful in regulating immune responses in pathophysiological situations that require DCs maturation (43).

SARS-CoV-2 stimulates IL-6 overproduction of alveolar macrophages through the toll-like receptor 4 (TLR4)-mediated NF-κB signaling pathway (111, 112). TLR4 is a receptor within the innate immune system, which recognizes pathogen-associated molecular patterns (PAMPs) of SARS-CoV-2 (3). AM3 is a TLR4 agonist (43). In the presence of AM3, TLR4/NF-kβ receptor would preferentially trigger p38 mitogen-activated protein kinase (p38MAPK) as a consequence of NF-kβ activation, which plays an essential capacity in the stimulation of immature DCs (43). Which would displace at least partially IL-6 production of alveolar macrophages mediated the TLR4/NF-kβ receptor. In other words, AM3 binds to the receptor TLR4/NF-kβ and prevents the SARS-CoV-2 from developing its effect. By increasing the AM3 concentrations, the effect could be achieved (competitive agonist). This would reduce IL-6 production and dampen exacerbated inflammation that leads to acute lung injury (113).

The diverse immunopathology caused by the COVID-19 infection could be a consequence of the interaction of the SARS-CoV-2 Spike (S) protein with the DC-SIGN receptor in respiratory DCs. DC-SIGN has been shown to mediate the binding of the SARS-CoV S protein to human DC with absorption into the endosome, followed by polarization of the endosome and presentation of the virus at an infectious synapse. Similarly, Human Immunodeficiency Virus (HIV) establishes the infectious synapse between T cells and DCs mediated by DC-SIGN (103). For this reason, a strategy to control the infection by COVID-19 could result in inhibiting initial stages of infection and dissemination of pathogens in the DCs through DC-SIGN, as it has been demonstrated by the oral AM3 administration (44) AM3 acts, dose dependently, on MDDCs by blocking the adhesion of pathogens such as Candida spp., Aspergillus spp. and Leishmania spp. by the interaction of AM3 on the activity of DC-SIGN of MDDCs. In relation to HIV, AM3 inhibits the function of DC-SIGN as a regulator of cell adhesion by blocking its binding to ICAM-3, which suppresses the binding of HIV gp120 to DC-SIGN in DCs. AM3 overrides the capacity of cells that express DC-SIGN to trap and transmit HIV viruses with replicative activity (44). Thus, it is expected that the application of AM3 would be beneficial against immunopathology caused by the COVID-19 infection because AM3 directly influences pathogen recognition by DCs altering functional capabilities of DC-SIGN. This could establish a therapeutic approach to early-stage SARS-CoV-2 infection.

Might AM3 be a Potential Adjuvant in the Vaccination Against SARS-CoV-2?

SARS-CoV-2 vaccines come in several types: whole-virus, subunit, attenuated, viral vectors, and nucleic acids; most are based on protein subunits (114). As of May 2021, fifteen vaccines have been approved for at least one country and thirty-three are in phase III in clinical trials (115). There are three vaccines approved by the FDA and [Pfizer-BioNTech, Moderna, and Janssen Pharmaceutical Companies of Johnson & Johnson (J&J's)] and another has been approved by the European Medicines Agency (EMA) in the European Union (Oxford/AstraZeneca). Other vaccines, such as the Novavax vaccine is expected to be approved in the next few months (116, 117).

BionTech/Pfizer have jointly developed BNT162b2 as an mRNA vaccine against SARS-CoV-2. The mRNA causes the host-cell to produce S-antigen proteins to stimulate an immune response (118). The efficacy has been demonstrated in clinical trials among participants with and without evidence of prior SARS-CoV-2 infection. Subjects who received the full, two-dose vaccine regime showed an approximately 95% efficacy rate after a median follow-period up of 2 months (119).

The mRNA-1273 vaccine, Moderna's COVID-19 vaccine, is a messenger RNA vaccine (118). It has been shown to have an efficacy of 94.1%, also based on a median follow-up of two months. The high efficacy was maintained across all age groups

(above 18 years) and was not affected by gender or ethnicity (120). AstraZeneca's COVID-19 (AZD1222) vaccine (C19VAZ), formerly known as ChAdOx1 nCoV-19, is made from a virus (ChAdOx1), a weakened version of a common cold virus (adenovirus). Genetic material has been added to the ChAdOx1 construct, which is used to make the SARS-CoV-2 coronavirus proteins called Spike (S) glycoprotein (118). The efficacy demonstrated in clinical trials in participants who received the full vaccine series (2 doses), regardless of the interval between doses, was 63.1%, based on a median follow-up of 80 days, but with a tendency to increase the longer gap between doses (121). J&J's vaccine (Ad26.COV2.S) is based on incompetently replicating recombinant adenovirus serotype 26 (Ad26) vector, encoding a stabilized, full-length SARS-CoV-2 spike protein (118). J&J's vaccine was 66.3% effective in clinical trials in preventing confirmed COVID-19 disease in persons with no evidence of previous infection two weeks after receiving the vaccine. Individuals achieved maximum possible protection within two weeks after vaccination (122). Although Novavax's vaccine (NVX-CoV2373) has not been approved yet, its Phase 3 clinical trial conducted in in the United Kingdom (UK) has demonstrated promising results with 89.3% of efficacy (118), even against the UK and South African variants (123).

AM3 has shown to be a useful adjuvant in hepatitis B vaccination in healthy people who previously did not develop Hepatitis B surface antigen (HBsAg) > 10 IU/ml titers in response to recombinant hepatitis B vaccine (34). Also, oral administration of AM3 (3 g/d), in patients with advanced renal disease and undergoing hemodialysis who did not respond to hepatitis B vaccination, for 30 consecutive days beginning 15 days before the first dose of vaccine maintained protective titers until six months after the final dose of vaccine. But not in the control group (33). In addition, the percentage of patients with high response (anti-HBsAg > 100 IU/L) and medium anti-HBsAg titers in the AM3 group was significantly higher than in the placebo group (33). This demonstrates that AM3 is a safe and easily tolerated oral agent that boosts long-term serological immunity to hepatitis B by developing prolonged protective anti-HBsAg titers that allow for long-term serological immunity after vaccination.

It is believed that most of the new COVID-19 vaccines may be low in immunogenicity in humans (114). Relative to previous studies (33, 34), administration of the AM3 immunomodulatory may result in a clear and prolonged antibody response that flows to SARS-CoV-2 vaccination. The significant increase in the percentage of patients with protective anti-HBsAg antibody titers and an increase in the rate of naive patients who maintain a protective response after six months of follow-up, induced by the adjuvant use of AM3 (33, 34), could support AM3 as an adjuvant to COVID-19 vaccination.

Although the mechanism is unknown, the stimulation and conservation of antibody titers observed are likely due to the establishment of long-term immune memory due to the influence of enhanced cell-mediated immunity function (124). The cell-mediated immunity function is considered the main mechanism of protecting and eliminating intracellular infectious agents, especially viral infections (125).

EFFECT OF AM3 ON BIOCHEMICAL MUSCULAR DAMAGE MARKERS

Muscle damage includes: 1) increased muscle proteins in the blood creatine kinase (CK), lactate dehydrogenase (LDH), and myoglobin (Mb); 2) decreased force generation; 3) capacity inflammatory cell infiltration; 4) disruption of Z-disks (delineate the lateral borders of sarcomeres which are the smallest functional units in skeletal muscle) and cell membrane damage (126, 127). Alterations in the immune system after strenuous exercise of athletes have as a consequence pathological changes in tissues comparable to other diseases such as bacterial sepsis or viral infections (128). In athletes, the inflammatory response is associated with significantly increased muscle proteins such as CK, Mb and LDH in blood (127).

As COVID-19 has demonstrated that cytokine release syndrome (CRS) produces an uncontrolled and overwhelming release of pro-inflammatory and inflammatory facilitators. The cytokine concentration was increased in COVID-19 patients and can be used as a predictive factor of disease severity in patients with COVID-19 (129). This situation produces breakdown of muscular fiber and connective tissue (108). Ultrastructural damage of muscle tissue is a potential complication arising from COVID-19-associated inflammatory cytokine production and release causing severe muscle injury (130). Muscle fibers contain proteolytic enzymes that, upon injury, are released and initiate degradation of lipid and protein structures of the injured cell. The rapid breakdown of damaged muscle fibers and connective tissue is accompanied by diffusion of intracellular components into the plasma (127). Patients with muscle weakness, myalgia, muscle atrophy, myositis, and rhabdomyolysis have been observed with some of the symptoms most commonly reported by patients with COVID-19. All of them had elevated serum levels of CK, Mb, LDH, and elevated serum levels of CRP and FER (131).

In a study conducted on basketball players (30), the placebo group experienced significant increases in CK, Mb and LDH after 30 days of exercise practice. The supplementation of AM3 in the experimental group, not only inhibited these changes, but also resulted in a significant decrease from baseline in serum concentrations of CK, Mb and LDH. AM3 prevents the changes of muscular damage biomarkers after exercise in elite athletes (29). Cordova et al. (25, 31) have reported that AM3 supplementation of 5 g/day during 6 weeks reduce plasma levels of enzyme activities associated with muscle damage. Therefore, the use of AM3 in patients with COVID-19 could be an effective therapeutic alternative to attenuate muscle damage (**Figure 2**) but also in post-COVID-19 recovery.

The muscular biomarkers that may provide information on the time course of COVID-19 are based on clinical findings associated with SARS, MERS and other viral respiratory infections (132). Variations in the biomarkers of muscle damage such as CK, Mb and LDH may help predict the course of COVID-19-associated pneumonia. Severe infections produce tissue damage and organ failure that play a more important role in SARS-CoV-2 through cytokines and release of CK, Mb and LDH (132–135). In this way, the biochemical results of muscle damage found in SARS-CoV-2 infected patients show that the muscle damage marker CK was

elevated in 14% of COVID-19 patients (134). Mb elevations were more pronounced in severe patients admitted to ICU *vs.* non-severe hospitalized patients with COVID-19 (104). With respect to LDH, a 6-fold and 16-fold increase in LDH increased the likelihood of severe symptoms and increases in mortality and morbidity in COVID-19 patients (132, 135).

CONCLUSION

AM3, a polysaccharide/protein compound, is a naturally occurring oral immunomodulatory compound. The AESAN categorizes AM3 as "Food Supplement" with regulatory effects on the immune system. AM3 has shown a wide range of regulatory effects on innate and adaptive immunity in experimental and clinical models of inflammation and disease. AM3 is an effector of immune-cell activity involved in response to SARS-CoV-2. AM3 activates the NKC production, positively modulates IFN secretion, promotes PBMC activity, and reduces inflammatory cytokine production. AM3 also reduces muscle, hepatic and pulmonary damage, decreases circulating NO levels. To date, no study has been reported neither side effects nor toxicity of the use of AM3. However, in future clinical trials, a pharmacovigilance appendix would be desirable in infections such as COVID-19 that frequently involve hyper-inflammatory responses. Clinical studies of AM3 in COVID-19 patients are needed to assess whether AM3 may be a preventive and therapeutic strategy in COVID-19 infections. Also, AM3 may be a useful adjuvant to SARS-CoV-2 vaccination.

AUTHOR CONTRIBUTIONS

Conceptualization, DF-L and CF-L. Methodology, DF-L, CF-L, and JM-A. Writing—original draft preparation, DF-L and CF-L. Writing—review and editing, DA, JM-A, JGH, and MG-G. Visualization, CF-L, DPA, JG, and MG-G. Supervision, DF-L. All authors contributed to the article and approved the submitted version.

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The Induction of Alpha-1 Antitrypsin by Vitamin D in Human T Cells Is TGF-β Dependent: A Proposed Anti-inflammatory Role in Airway Disease

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Background: Vitamin D upregulates anti-inflammatory and antimicrobial pathways that promote respiratory health. Vitamin D synthesis is initiated following skin exposure to sunlight, however nutritional supplementation can be required to address deficiency, for example during the winter months or due to cultural constraints. We recently reported that 1α,25-dihydroxyvitamin D3 (1,25$(OH)_2$D3) treatment induced alpha-1 antitrypsin (AAT) expression in CD4+, but not CD8+ T cells, with evidence supporting an immunoregulatory role.

Research Question: To understand the relationship between vitamin D, lung AAT levels and T lymphocytes further we investigated whether TGF-β is required as a co-factor for 1,25$(OH)_2$D3-induced upregulation of AAT by vitamin D in CD8+ T cells *in vitro* and correlated circulating vitamin D levels with lung AAT levels *in vivo*.

Results: 1,25$(OH)_2$D3 in combination with TGF-β1 increased AAT expression by CD8+ T cells, as well as *VDR* and *RXRα* gene expression, which may partly explain the requirement for TGF-β. CD4+ T cells may also require autocrine stimulation with TGF-β as a co-factor since 1,25$(OH)_2$D3 was associated with increased TGF-β bioactivity and neutralisation of TGF-β partially abrogated 1,25$(OH)_2$D3-induced *SERPINA1* gene expression. Neither CD4+ nor CD8+ T cells responded to the circulating vitamin D precursor, 25-hydroxyvitamin D3 for induction of *SERPINA1*, suggesting that local generation of 1,25$(OH)_2$D3 is required. Transcriptional gene profiling studies previously demonstrated that human bronchial epithelial cells rapidly increased TGF-β2 gene expression in response to 1,25$(OH)_2$D3. Here, human epithelial cells responded to precursor 25(OH)D3 to increase bioactive TGF-β synthesis. CD8+ T cells responded comparably to TGF-β1 and TGF-β2 to increase 1,25$(OH)_2$D3-induced AAT.

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However, CD8+ T cells from adults with AAT-deficiency, homozygous for the Z allele of *SERPINA1*, were unable to mount this response. AAT levels in the airways of children with asthma and controls correlated with circulating 25(OH)D3.

Conclusions: Vitamin D increases AAT expression in human T cells and this response is impaired in T cells from individuals homozygous for the Z allele of *SERPINA1* in a clinic population. Furthermore, a correlation between circulating vitamin D and airway AAT is reported. We propose that vitamin D-induced AAT contributes to local immunomodulation and airway health effects previously attributed to vitamin D.

Keywords: vitamin D, T cell, alpha-1 antitrypsin (AAT), anti-inflammatory, HBEC, airway disease

INTRODUCTION

Vitamin D is increasingly recognised as an important factor in regulating immunity and respiratory health, and vitamin D deficiency is associated with several immune-mediated airway diseases, including asthma and chronic obstructive pulmonary disease (COPD) (1–3). This is attributed to its diverse immuno-modulatory functions including the capacity to promote anti-microbial pathways, suppress inflammatory responses while enhancing immuno-modulatory functions such as anti-inflammatory IL-10 production (4, 5).

Alpha-1 antitrypsin (AAT) plays a critical role in protecting lung parenchymal tissue from direct elastinolytic degradation and pro-inflammatory effects of serine proteases, notably neutrophil elastase (6). It is predominantly synthesized in the liver by hepatocytes and is secreted into the plasma as the most abundant circulating antiprotease. The metastable structure of native AAT and other members of the serine protease inhibitor (serpin) superfamily provides the potential for stabilizing conformational change (7). This is utilized in the functional mechanism of protease inhibition, but also renders AAT vulnerable to point mutations, as seen in the Glu342Lys (Z) variant. Within hepatocytes, that are the major source of systemic AAT, this triggers misfolding in the endoplasmic reticulum, aberrant conformational change and stabilizing intermolecular linkage to form AAT polymers (8). The results are a deficiency of circulating functional AAT, and retention of misfolded and polymerized AAT within hepatocytes associated with pro-inflammatory and pro-fibrotic gain-of-function (9). This combination predisposes Z variant homozygotes (denoted Pi*ZZ) to severe and early-onset COPD and liver disease (hepatitis, cirrhosis, and hepatocellular carcinoma) (10). The lung disease seems predominantly driven by loss-of-function mechanisms though extracellular polymers encourage neutrophil chemotaxis and activity and are found in the circulation and lung (11).

AAT displays multiple immuno-regulatory functions including inhibition of neutrophil chemotaxis (12), induction of IL-1Ra by macrophages (13) and the development of tolerogenic dendritic cells and their production of IL-10 (14, 15). We have recently shown the requirement of AAT in the $1,25(OH)_2D3$-mediated induction of IL-10 in CD4+ T cells (16). In addition to immunomodulatory effects mediated by antiprotease function, immunomodulatory effects of AAT that are independent of antiprotease function are now recognized (17). The absence of these effects may further exacerbate the pro-inflammatory state in lungs of individuals with AAT deficiency.

Cells within the lungs e.g., alveolar epithelial cells, monocytes and neutrophils are capable of secreting AAT to supplement liver-derived AAT in the airways (18–20). We have demonstrated that the active form of vitamin D, $1,25(OH)_2D3$, upregulates expression of *SERPINA1*, the gene encoding the serine protease inhibitor AAT, and AAT protein secretion by human primary CD4+ T cells (16). Conversely this was not seen for monocytes. Moreover, vitamin D reduces secretion of matrix metalloproteinase (MMP-)9 that degrades AAT (21, 22). These data suggest that vitamin D availability may boost AAT levels within the airway microenvironment and so represent an additional mechanism by which vitamin D protects the airways.

Our published data demonstrate that $1,25(OH)_2D3$ alone does not upregulate AAT in human CD8+ T cells (16). Given the proposed role of CD8+ T cells in chronic airway conditions such as COPD (23), we investigated whether a co-factor that acts in concert with $1,25(OH)_2D3$ may induce AAT synthesis by human CD8+ T cells. TGF-β was investigated, since previous studies reported the capacity of hepatoma cell lines and human lung epithelial cell lines to produce AAT in response to TGF-β (24, 25). Furthermore, TGF-β and vitamin D are reported to act co-operatively. Independent studies report, for example, that TGF-β increases gene and protein expression for VDR (26) and the enzyme CYP27B1, which converts precursor vitamin D, 25(OH)D, to the active moiety, $1,25(OH)_2D3$ (27, 28). Furthermore, $1,25(OH)_2D3$ in combination with TGF-β increases the frequency of CD4+ Foxp3+ Treg cells (29). These data provide the rationale for the present study to investigate whether $1,25(OH)_2D3$ and TGF-β interact with each other for enhanced effects in human CD8+ T cells.

MATERIALS AND METHODS

Subject Characteristics

Healthy donors ("Human peripheral blood cell laboratory studies to investigate the role of immune and inflammatory pathways in respiratory disease" by the local research ethics committee, REC reference: 14/LO/1699) were recruited and provided full written informed consent with full Research Ethics Committee approval. COPD patients without AAT deficiency (Chest clinic, Guy's Hospital, 14/LO/1699), and PiZZ-AAT deficient patients with or

without COPD (Targeting dysfunctional mechanisms in alpha-1 antitrypsin deficiency, The Royal Free Alpha-1 clinic, REC reference: 13/LO/1085) were recruited and provided full written informed consent with full Research Ethics Committee approval, including anonymization of donors to researchers. The study in a pediatric asthma cohort and control children undergoing diagnostic bronchoscopy for non-asthma related purposes was approved by the Royal Brompton and Harefield Research Ethics Committee (09/H07008/48) which has been previously described Gupta et al. (30). Informed consent was obtained from parents and age-appropriate assent from children. Serum levels of 25-hydroxyvitamin D were measured as previously described by Gupta et al. (30). All work adhered strictly to institutional safety guidelines and procedures. **Table 1** provides data on the number of individuals studied throughout, and figure legends identify number of individual donors in each experimental series.

Cell Isolation and Culture

Human peripheral blood mononuclear cells (PBMCs) were isolated, as previously described Xystrakis et al. (31). CD4+ and CD8+ T cells were isolated using Dynabeads CD4 or CD8 positive selection kits (Invitrogen, Paisley, UK). Cells (1×10^6/ml) were cultured in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, and 50 μg/ml gentamicin, and stimulated with plate-bound anti-CD3 (1 μg/ml; OKT3) plus 50 IU/ml recombinant human IL-2 (Eurocetus, UK) in the presence or absence of $1,25(OH)_2D3$ (BIOMOL research labs, UK), TGF-β1 (R&D Systems, UK), TGF-β2 (R&D Systems) or anti-TGF-β (R&D Systems) at indicated concentrations for 7 days. NIST SRM1648a Urban Particulate Matter (National Institute of Standards & Technology, USA) is an urban total particulate matter reference material with mean particle diameter 5.85 μm, collected in the USA. NIST was prepared as previously described by Pfeffer et al. (32).

HBEC Isolation and Culture Conditions

Primary human bronchial epithelial cells (HBECs) were acquired and maintained as previously described Pfeffer et al. (33). HBECs were stimulated with 50 μg SRM1648a, a standard reference urban particulate matter, NIST, in the absence or presence of 10, 100, or 1,000 nM 25(OH)D3 for 24 h. Lysed cell monolayers were collected for assessment by qRT-PCR. HBECS were also stimulated with 25(OH)D3 (1 μM) for 48 h and culture supernatant collected for TGF-β bioassay.

qRT-PCR

RNA was extracted from cell pellets using the Rneasy Mini kit (Qiagen, Crawley UK) according to the manufacturer's instructions. Nanodrop ND-1000 spectrophotometer was used to quantify RNA. 250 ng of RNA was reverse transcribed into cDNA. Quantitative real-time PCR was performed, as previously described Urry et al. (34) in triplicates by using an Applied Biosystems 7900 HT system and FAM-labeled Assay-on-Demand reagent sets for *SERPINA1* (Hs01097800_m1), *VDR* (Hs01045846_m1), *RXRα* (Hs01067640_m1). Quantitative real-time PCR reactions were multiplexed with VIC-labeled 18S primers and probes (Hs99999901_s1) as an endogenous control and analysed with SDS software, version 2.1 (Applied Biosystems, Foster City, Calif), according to $2^{-\Delta Ct}$ ($\times 10^6$).

AAT ELISA

The AAT ELISA employed a commercial polyclonal rabbit anti-human AAT primary capture antibody (Dako, UK) and a biotin-conjugated rabbit anti-human AAT secondary detection antibody [in-house purified, previously described in Dimeloe et al. (16)]. Standards were serially diluted in RPMI medium plus 10% foetal calf serum, by serial 1:2 dilutions from the top standard of 200 ng/ml using human plasma-purified AAT (Sigma-Aldrich, UK). Streptavidin-alkaline phosphatase (Sigma-Aldrich, UK) and 4-Nitrophenyl phosphate disodium salt hexahydrate (1 mg/ml; Sigma-Aldrich, UK) were used to detect AAT. Absorbance was measured at 405 nm on an Anthos HTII plate reader (Anthos, UK) using Softmax pro software and quantified using GraphPad Prism version 6 (GraphPad software Inc., USA). The lower limit of detection was 0.32 ng/ml.

TGF-β Bioassay

TGF-β bioactivity was measured using mink lung epithelial cell lines (MLECs) transfected with plasminogen activator inhibitor-1, a TGF-β target gene, fused with the firefly luciferase construct (kindly donated by Professor Daniel Rifkin, New York University). Briefly, 3×10^5 MLECs/ml were incubated for 14 h at 37°C in 5% CO_2 with cell culture supernatants or standards. Culture media was aspirated, washed, cells lysed and luciferase activity determined using Firefly Luciferase Assay Kit (Biotium, USA) and measured by luminescence (1450 MicroBeta TriLux; PerkinElmer, USA) in accordance with the manufacturer's instructions. Light emitted corresponds to the amount of bioactive TGF-β. Serum free RPMI was used for these experiments.

Data Analysis

Data are shown as mean ± standard error of mean (SEM) unless otherwise indicated.

Data analysis was performed using Graphpad Prism version 6.00 for Mac OS X (Graphpad software Inc., USA). Statistical test used as in relevant figure legend.

RESULTS

Exogenous TGF-β1 and $1,25(OH)_2D3$ Act Cooperatively to Enhance *SERPINA1*/AAT in CD8+ T Cells

Human CD4+ T cells, but not CD8+ T cells, treated with $1,25(OH)_2D3$ alone increase *SERPINA1* gene expression and AAT protein secretion (16). The requirement for TGF-β as a cofactor to promote synthesis of AAT by CD8+ T cells was investigated. Peripheral blood CD4+ and CD8+ T cells were stimulated with anti CD3/IL-2 in the presence of 10 nM or 100 nM $1,25(OH)_2D3$, with or without the addition of TGF-β1 over a broad range titration (0.02–20 ng/ml) (**Figure 1**). After 7 days in culture both 10 and 100 nM $1,25(OH)_2D3$ significantly increased *SERPINA1* transcription and 100 nM $1,25(OH)_2D3$ significantly upregulated AAT secretion in CD4+

TABLE 1 | Clinical parameters of healthy control, COPD, and PiZZ patients.

CD4+ T cells	Healthy	COPD	PiZZ	CD8+ T cells	Healthy	COPD	PiZZ
N number	6	9	6	N number	12	9	20
Gender (F:M)	4:2	5:4	4:2	Gender (F:M)	6:6	5:4	9:11
Age range	24–58	53–79	18–62	Age range	26–63	53–79	18–75
Lung function				Lung function			
FEV1%	Expected to be normal	28–94	27–120	FEV1%	Expected to be normal	28–94	27–125
FEV1/FVC %		27–69	39–81	FEV1/FVC %		27–69	20–83

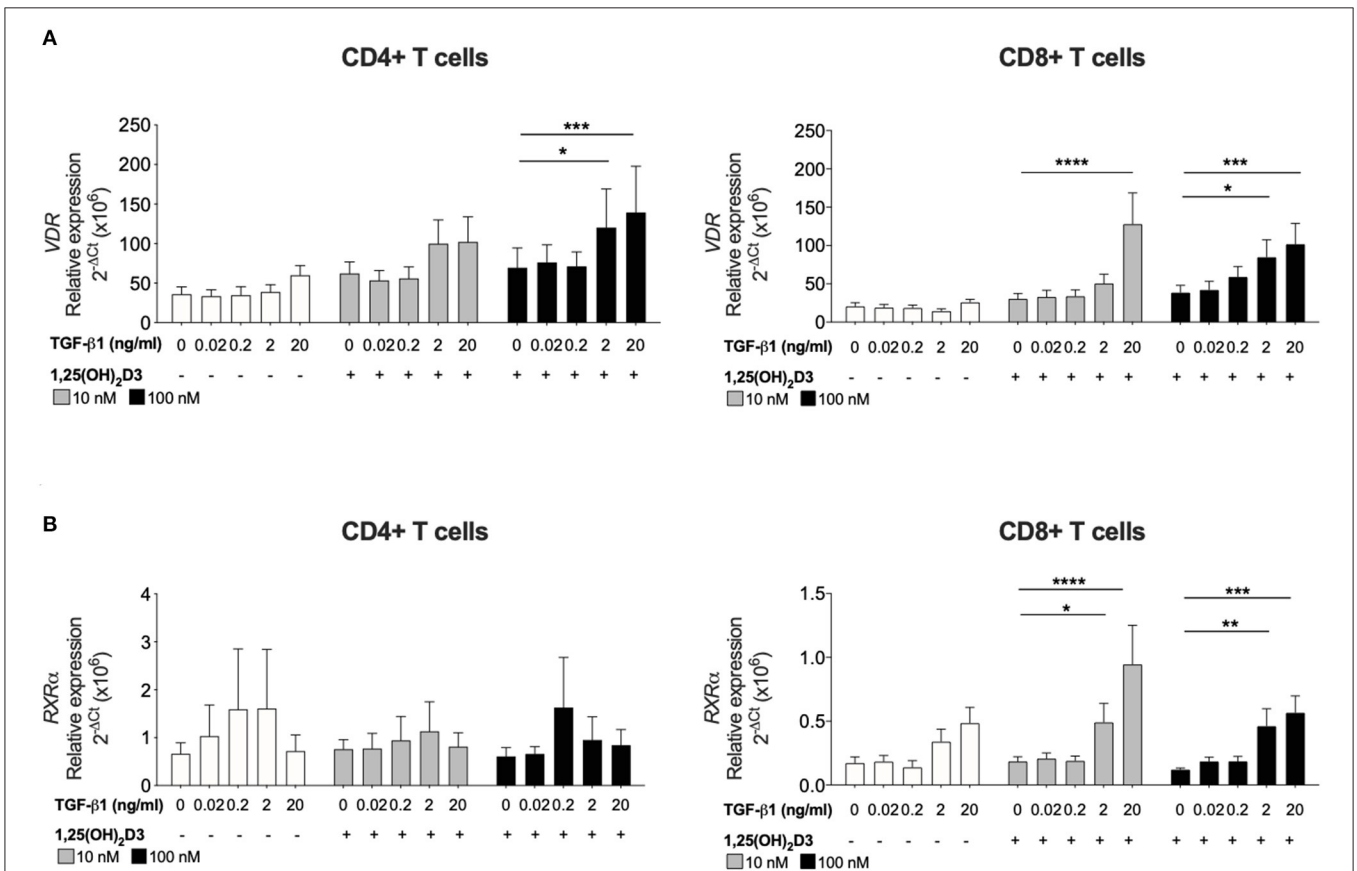

FIGURE 1 | TGF-β1 together with 1,25(OH)$_2$D3 upregulates *VDR* and *RXRα* gene expression by CD8+ T cells. CD4+ and CD8+ T cells ($n = 6$) were stimulated and cultured for 7 days in the absence or presence of 10 or 100 nM 1,25(OH)$_2$D3, with or without TGF-β1 (0.02, 0.2, 2, 20 ng/ml). Gene expression of **(A)** *VDR* and **(B)** *RXRα* gene expression was quantified by qRT-PCR. Data are expressed as mean ± SEM and statistical analysis employed a 2-way ANOVA with Bonferroni post-tests with $^{*}p < 0.05$, $^{****}p < 0.0001$.

T cells, but not in CD8+ T cell cultures. Addition of 2 or 20 ng/ml TGF-β1 together with the higher concentration of 1,25(OH)$_2$D3 significantly increased both *SERPINA1* and AAT expression in CD8+ T cell cultures to levels similar to those observed in CD4+ T cells treated with the same concentration of 1,25(OH)$_2$D3. However, the addition of TGF-β1 alone had no effect, whilst TGF-β1 with 1,25(OH)$_2$D3 had no additional effect in CD4+ T cell cultures. The physiological concentration of TGF-β has been reported to be up to 4 ng/ml in human plasma (35), we therefore have selected 2 ng/ml to proceed in the study.

TGF-β1 and 1,25(OH)$_2$D3 Act Together to Upregulate *VDR* and *RXRα* Expression in CD8+ T Cells

To investigate how TGF-β1 modulates the response of CD8+ T cells to 1,25(OH)$_2$D3, the effect of TGF-β1 on *VDR* and *RXRα* was assessed since the VDR-RXRα complex binds 1,25(OH)$_2$D3 and is essential for subsequent vitamin D-mediated effects. Addition of 1,25(OH)$_2$D3 alone to cultures of CD4+ or CD8+ T cells had no effect on the gene expression of either receptor. Inclusion of TGF-β1 at higher doses (2 and 20 ng/ml) significantly enhanced 1,25(OH)$_2$D3-induced

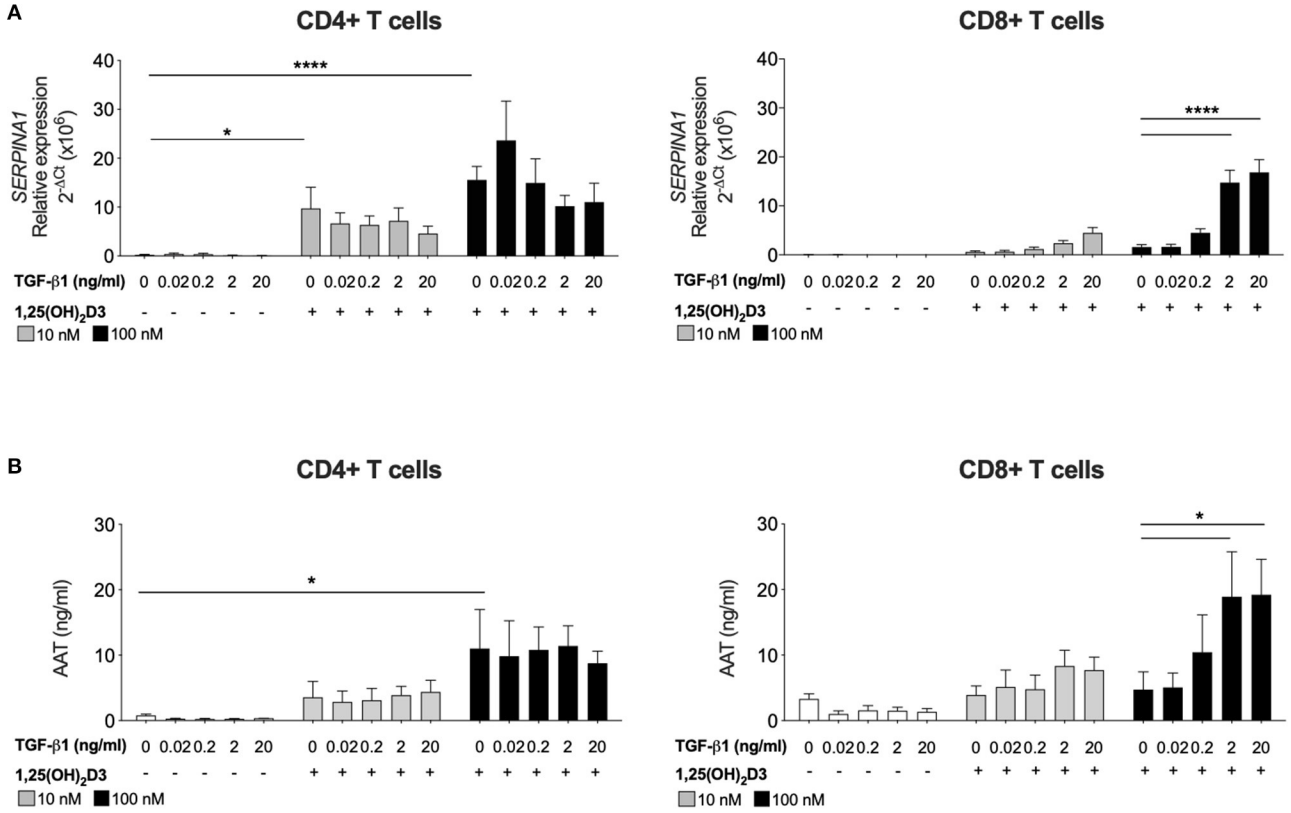

FIGURE 2 | CD8+ T cells require exogenous TGF-β1 to enhance $1,25(OH)_2D3$-induced *SERPINA1*/AAT. CD4+ and CD8+ T cells ($n = 6$) were stimulated and cultured for 7 days in the absence or presence of 10 or 100 nM $1,25(OH)_2D3$, with or without TGF-β1 (0.02, 0.2, 2, 20 ng/ml). **(A)** Gene expression of *SERPINA1* was quantified by qRT-PCR. **(B)** Culture supernatants were assessed for AAT protein secreted by ELISA. Data are expressed as mean ± SEM and statistical analysis employed a 2-way ANOVA with Bonferroni post-tests with $^*p < 0.05$, $^{****}p < 0.0001$.

(100 nM) *VDR* in both CD4+ and CD8+ T cells (**Figure 2A**). Expression of *RXRα* was not significantly affected in CD4+ T cells, the combination of $1,25(OH)_2D3$ and TGF-β1 significantly enhanced *RXRα* expression in CD8+ T cell cultures (**Figure 2B**).

Requirement for Endogenous or Exogenous TGF-β in $1,25(OH)_2D3$-Mediated Regulation of *SERPINA1*

Since CD4+ T cells did not show the same requirement for exogenous TGF-β as CD8 + T cells for *SERPINA1*/AAT induction, the issue of whether this was due to their greater endogenous production of bioactive TGF-β in comparison to CD8+ T cells was examined. Bioactive TGF-β secretion was quantified in parallel cultures of CD4+ and CD8+ T cells, using mink lung epithelial cells that were transfected with a TGF-β target gene plasminogen activator inhibitor 1 and a luciferase assay. In the absence of $1,25(OH)_2D3$, there was no difference in bioactive TGF-β levels between CD4+ and CD8+ T cells at any time point. However, bioactive TGF-β was elevated in supernatants harvested from CD4+ T cells after 5 and 7 days of culture with 100 nM $1,25(OH)_2D3$. No effect was observed in CD8+ T cells (**Figure 3A**).

In a complementary approach, CD4+ and CD8+ T cells were cultured in the presence of neutralising TGF-β or isotype control antibodies to assess the role of endogenous, autocrine TGF-β in controlling *SERPINA1* expression. Anti-TGF-β significantly down-regulated $1,25(OH)_2D3$-mediated *SERPINA1* expression in CD4+ T cell cultures, but in parallel CD8+ T cell cultures, $1,25(OH)_2D3$ did not significantly increase *SERPINA1*, and neutralization of TGF-β did not markedly modify this response (**Figure 3B**). However, modulation of AAT protein expression by neutralization of TGF-β did not achieve statistical significance (data not shown). Together, these observations indicate that the $1,25(OH)_2D3$-mediated induction of *SERPINA1* in both CD4 and CD8 T cells is at least partially TGF-β dependent. CD4+ T cells appear capable of autocrine priming with TGF-β whilst CD8+ T cells require exogenous addition of this co-factor.

Human T Cells Respond to $1,25(OH)_2D3$ but Not Its Circulating Precursor 25(OH)D3

The studies presented here and in our earlier publication (16) have assessed the capacity of active vitamin D ($1,25(OH)_2D3$) to enhance *SERPINA1*/AAT production by T cells. Vitamin D circulates predominantly in the precursor form 25(OH)D3, which has a far longer half-life and is found at ∼1,000-fold higher

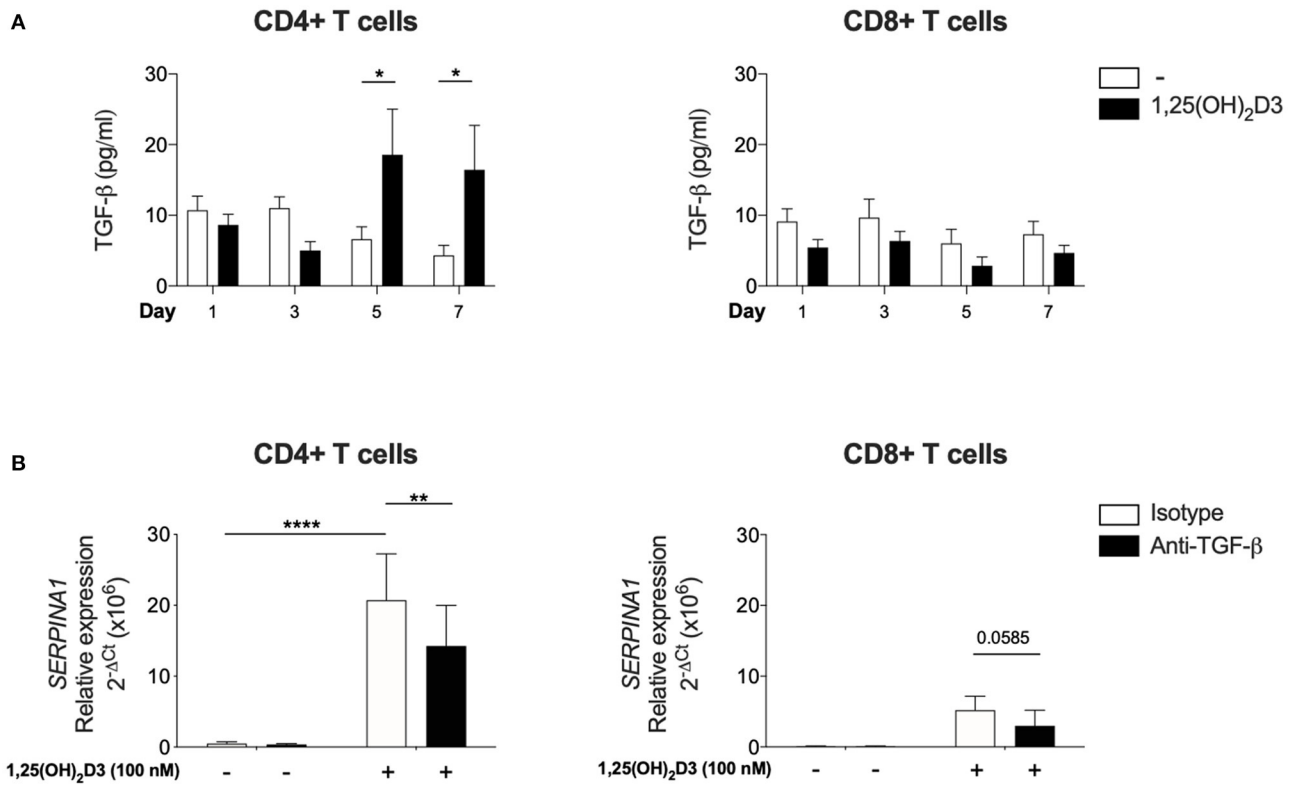

FIGURE 3 | Significant upregulation of bioactive TGF-β is observed with $1,25(OH)_2D3$ treatment in CD4+ T cells and $1,25(OH)_2D3$ induction of *SERPINA1*/AAT is partially inhibited by anti-TGF-β. CD4+ and CD8+ T cells ($n = 6$) were stimulated and cultured in the absence or presence of 100 nM $1,25(OH)_2D3$. **(A)** Supernatant from indicated time points were harvested and TGF-β bioactivity was measured using transfected mink epithelial lung cells and luciferase activity (luminescence, as relative light unit corresponds to TGF-β bioactivity), and converted to pg/ml. **(B)** Cells were cultured with 100 nM $1,25(OH)_2D3$ in the presence of anti-TGF-β or an isotype control for 7 days. $1,25(OH)_2D3$-mediated induction of *SERPINA1* expression was assessed by qRT-PCR. Data are expressed as mean ± SEM and statistical analysis employed a 2-way ANOVA with Bonferroni post-tests with $^*p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$.

concentrations. CYP27B1, the Vitamin D 1-alpha-hydroxylase enzyme which converts 25(OH)D3 to $1,25(OH)_2D3$, is expressed by CD4+ T cells providing the potential for local modulation of vitamin D activity. However, the degree to which these cells are able to generate physiologically relevant concentrations of the active form remains controversial (36, 37). CD4+ and CD8+ T cells were therefore stimulated in the presence of 25(OH)D3 or $1,25(OH)_2D3$, with or without 2 ng/ml TGF-β. Upregulation of *SERPINA1* was observed in the presence of $1,25(OH)_2D3$ in the CD4+ T cell cultures, with much lower levels seen in the CD8+ T cells. Neither cell type responded to 25(OH)D3 for upregulation of *SERPINA1* (**Figure 4A**), suggesting that T cells may require $1,25(OH)_2D3$ generated locally by other cell types to upregulate *SERPINA1 in vivo*.

Human Bronchial Epithelial Cells May Provide a Local Source of Active Vitamin D to Control T Cell Responses

Human bronchial epithelial cells (HBECs) are known to respond to both 25(OH)D3 and $1,25(OH)_2D3$ (32, 38) and are considered an important source of local mediators that activate immune cells in the airways (32, 39). In a recent study using transcriptional gene profiling we reported that human primary HBECs stimulated with $1,25(OH)_2D3$ increased *TGFB2* gene expression (32). Here, this observation was extended to demonstrate that primary HBECs increase *TGFB2* expression upon 25(OH)D3 stimulation in a concentration-dependent manner, when activated *in vitro* by exposure to NIST, a standard reference source of total urban particulate matter (SRM-1648a) which has been previously described (32) (**Figure 4B**). NIST was used as a relevant environmental stimulus of HBEC with previously observed effect on induction of inflammatory pathways (32). Increased bioactive TGF-β was evident at 48 h in HBEC culture supernatants in response to 1 μM 25(OH)D3 in culture (**Figure 4C**).

In order to support the concept that HBEC derived bioactive TGF-β may enable T cell responsiveness to $1,25(OH)_2D3$ for upregulation of *SERPINA1*/AAT, the capacity of TGF-β1 and TGF-β2 isoforms to enhance this response in CD8+ T cells was assessed. CD4+ and CD8+ T cells were stimulated in culture with 0 or 100 nM $1,25(OH)_2D3$ in the absence or presence of TGF-β1 or TGF-β2. Both TGF-β isoforms in the presence, but not the absence of $1,25(OH)_2D3$, significantly increased

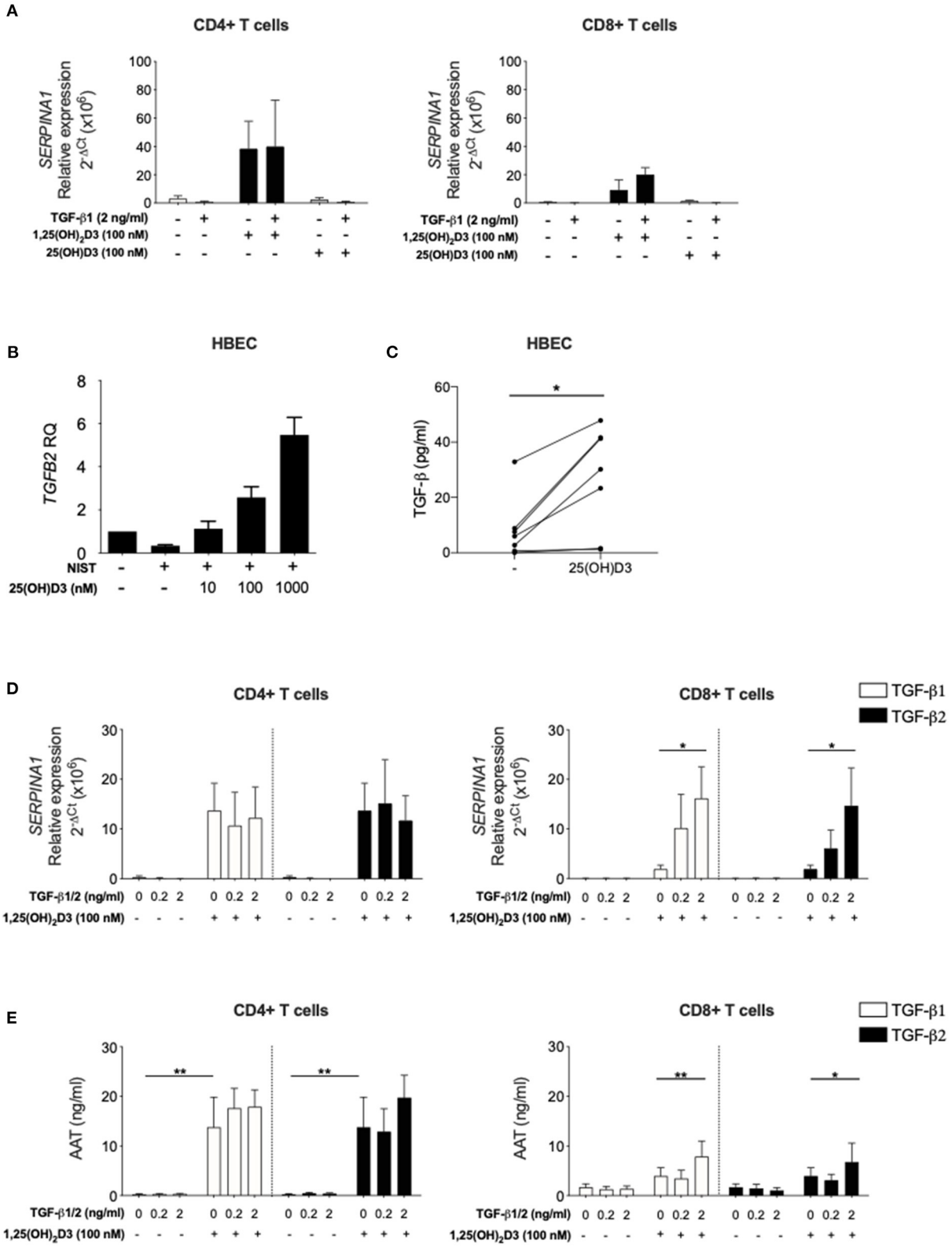

FIGURE 4 | Inactive vitamin D does not induce *SERPINA1* expression in T cells, but does induce production of bioactive TGF-β in HBECs. Both TGF-β isoforms upregulate 1,25(OH)$_2$D3-mediated *SERPINA1*/AAT induction in CD8+ T cells. **(A)** CD4+ and CD8+ T cells were isolated from peripheral blood of healthy donors

(Continued)

FIGURE 4 | ($n = 4$) and cultured for 7 days in the absence or presence of either 100 nM 1,25(OH)$_2$D3 or 100 nM 25(OH)D, with or without 2 ng/ml TGF-β1. Cell pellets were harvested and assessed for *SERPINA1* expression. **(B)** Human bronchial epithelial cells (HBEC, $n = 5$) were analysed for *TGFB2* gene expression following 24 h stimulation with NIST and different concentrations of 25(OH)D3 (10, 100, and 1,000 nM). **(C)** HBEC ($n = 7$) were assessed for their ability to produce bioactive TGF-β in response to 48 h 1 μM 25(OH)D3. Cells were cultured for 7 days in the presence or absence of 100 nM 1,25(OH)$_2$D3 with or without TGF-β1 or TGF-β2 (0.2, 2 ng/ml). **(D)** Cell pellets were assessed for *SERPINA1* gene expression ($n = 3$–6). **(E)** Culture supernatants were assessed for AAT protein secretion by ELISA ($n = 6$). Data are expressed as mean ± SEM and statistical analysis employed a 2-way ANOVA with Bonferroni post-tests with $^{*}p < 0.05$, $^{**}p < 0.01$.

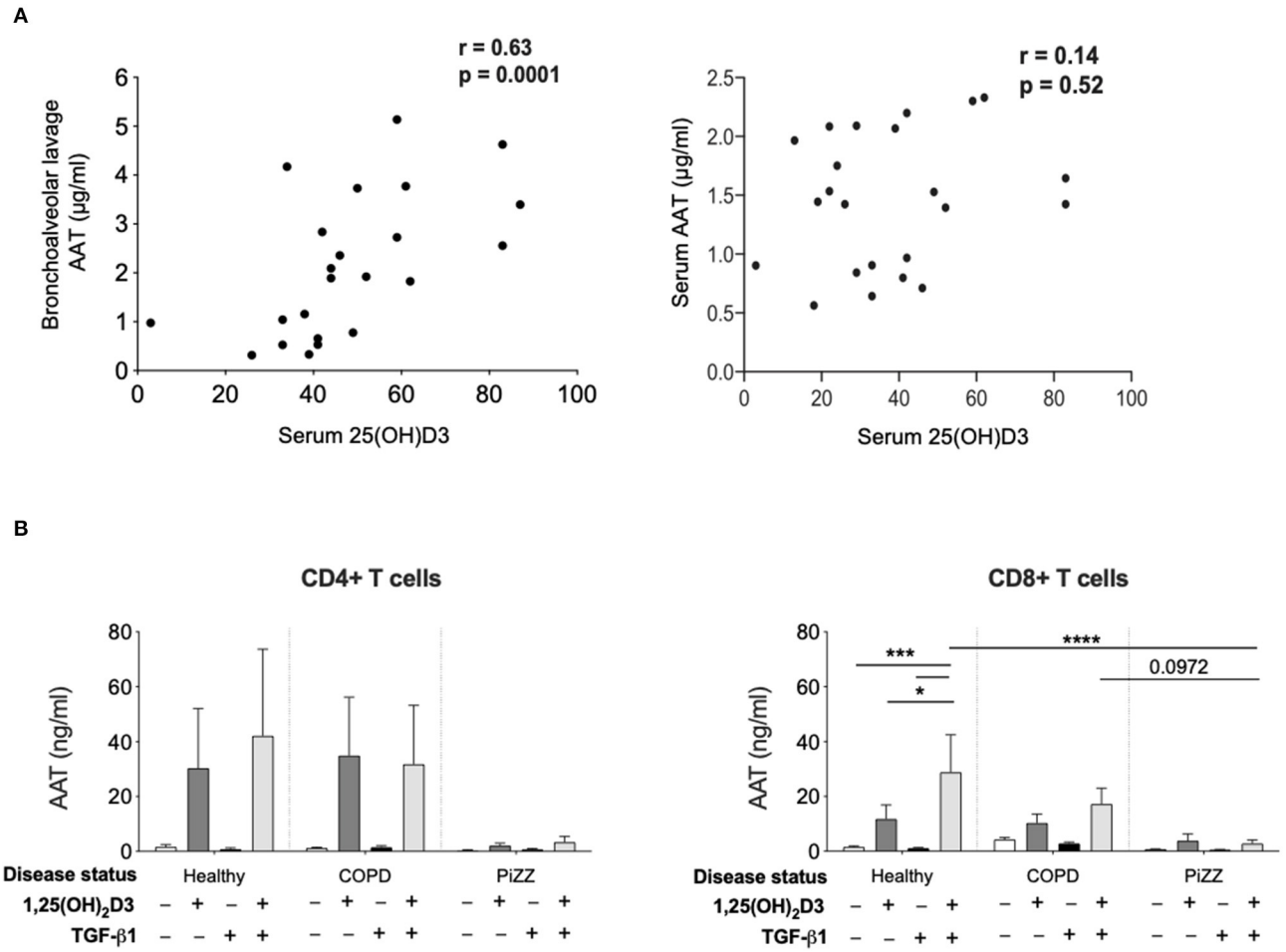

FIGURE 5 | Patient studies to assess *in vivo* relevance. **(A)** Serum concentration of 25(OH)D3, as well as bronchoalveolar lavage (left) and serum (right) concentration of AAT were assessed in a paediatric severe asthma cohort and in paediatric controls undergoing bronchoscopy. Correlations were assessed using Spearman's rank correlation. **(B)** CD4+ and CD8+ T cells were isolated from the peripheral blood of healthy donors (CD4+ $n = 6$, CD8+ $n = 12$), non-AAT deficient COPD ($n = 9$) and PiZZ patients (CD4+ $n = 6$, CD8+ $n = 20$) and cultured for 7 days in the absence (–) or presence of 1,25(OH)$_2$D3 (100 nM) with or without TGF-β1 (2 ng/ml) before culture supernatants were assessed for AAT protein secretion by ELISA. Data are expressed as mean ± SEM and statistical analysis employed a 2-way ANOVA with Bonferroni multiple comparison test with $^{*}p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

SERPINA1 expression and AAT production in CD8+ T cell cultures. As previously observed, CD4+ T cells responded to 1,25(OH)$_2$D3 with no further effect upon addition of either TGF-β preparation (**Figures 4D,E**).

Correlation Between Serum Vitamin D and Bronchoalveolar Lavage AAT Concentrations in Children With Asthma

Evidence to support the relevance of these experimental observations was explored in 2 distinct patient populations (**Figure 5**). At least one independent study reports a link between serum vitamin D levels and AAT levels in autoimmune disease, in this case type 2 diabetes (40). We previously described a clinically well-defined cohort of children with asthma and control children undergoing diagnostic bronchoscopies for non-asthma related investigations, in which lower vitamin D levels were associated with worse asthma control and lung function (30). Using archived serum and bronchoalveolar lavage from this cohort here we observed a significant positive correlation between serum 25(OH)D3 levels and AAT in the bronchoalveolar lavage but not the serum in this paediatric severe asthma cohort

(**Figure 5A**). These findings suggested that circulating vitamin D availability may contribute to control AAT synthesis locally in the airways, at least in part through effects on T cells.

Studies in Adults at Risk of COPD

Hepatocytes synthesise very high levels (1–2 g per day) of AAT, which is secreted into the circulation (41). Patients who are homozygous for the Z variant mutation (PiZZ genotype) in *SERPINA1*, produce AAT protein carrying a Glu342Lys substitution. This causes it to misfold, polymerise and aggregate within the hepatocyte such that only around 15% of normal levels of AAT protein are secreted, resulting in a similar circulating deficiency (9). These individuals represent up to 95% of the clinically diagnosed cases of AAT deficiency and are at greatly increased risk of early onset COPD. Whilst the situation in hepatocytes appears well-characterised, whether other cell types that produce lower levels of AAT will retain similar proportions of the synthesised protein as intracellular aggregates and demonstrate similarly profound secretion defects in the PiZZ context is not known. We therefore compared the levels of AAT secretion by CD4+ and CD8+ T cells treated with $1,25(OH)_2D3$ in cohorts of healthy controls, patients with COPD, and PiZZ AAT deficient individuals (**Figure 5B**). Clinical parameters for each group are summarized in **Table 1**. The AAT response was impaired in PiZZ CD4+ and CD8+ T cells (with or without TGF-β1). Simultaneous addition of $1,25(OH)_2D3$ and TGF-β1 significantly enhanced AAT secretion in healthy CD8+ T cells, and COPD CD8+ T cells showed a similar, albeit reduced trend for AAT secretion. However, PiZZ CD8+ T cells failed to secrete AAT in response to any of the treatments. Similar response profiles were observed in CD4+ cells between cohorts, but with greater variability. Overall, these observations strongly support defective secretion of AAT in PiZZ T cells, in response to stimuli that are able to increase AAT in T cells from healthy donors, consistent with the nature of the PiZZ defect in AAT secretion from hepatocytes (9). This may contribute to impaired local immunomodulation around T cells in PiZZ AAT deficiency.

DISCUSSION

A delicate balance between proteases and anti-proteases contributes to homeostasis in the lungs and several mediators, including vitamin D and AAT, contribute to this. This study highlights the following findings: (i) CD8+ T cells, in addition to CD4+ T cells, represent a cellular source for $1,25(OH)_2D3$-induced AAT in the airway microenvironment, (ii) TGF-β acts as a co-factor in this vitamin D controlled regulatory axis and (iii) this axis is defective in PiZZ patients with severe AAT deficiency. Based on this and our previous findings (16) we propose that T cell-derived AAT is likely to function as an intermediate for immunomodulatory roles of vitamin D in the airway (1, 5). The significant correlation between circulating levels of vitamin D with airway AAT levels in a pediatric cohort further supports a functional relationship between these two mediators *in vivo*. Our *in vitro* studies indicate this will be mediated by $1,25(OH)_2D3$ whilst implicating at least one further mediator upstream of the AAT response that we have not identified in our work to date. This work therefore provides an important starting point for further work to elucidate clear cascades of regulation and so better understand the relationship between vitamin D and AAT.

Hepatocytes are recognised as the major source of circulating AAT secreting 1–2 g/d into the circulation, however there is also good evidence for local synthesis, including in the airways (42, 43). The lower, but still significant levels of AAT secreted by T cells suggest that T cell derived AAT may act locally and have immunomodulatory functions, which have been reviewed elsewhere (44). In brief, AAT acts on structural and innate cells to increase anti-inflammatory mediators such as IL-1Ra, IL-10, to inhibit pro-inflammatory cytokine release, inhibit neutrophil chemotaxis and degranulation, and promote tolerogenic dendritic cells. In the adaptive arm, AAT increases the frequency of Foxp3 Treg cells and T cell synthesis of anti-inflammatory IL-10, whilst inhibiting pro-inflammatory mediators as well as B cell proliferation and autoantibody production. Many of these data arise from animal models where AAT is reported to control autoimmune disease, help to prevent transplant rejection and elastase-induced emphysema (45–48). Importantly, human intervention studies that build on these data are now emerging [reviewed by Song (49)]. Two recent examples report concomitant clinical and parallel immune readouts. AAT suppressed experimental graft vs. host disease (GVHD) by downmodulating inflammation, and AAT infusion was subsequently studied for treatment of steroid-resistant acute GVHD in humans. Magenau et al. reported a durable clinical response in refractory acute GVHD and that AAT administration appeared safe, with low rates of infection and with increasing ratios of T regulatory to effector T cells (50). Campos et al. (51) used double dose AAT infusion in AAT-deficient individuals to restore circulating AAT levels within the normal range, as well as to reduce serine protease activity and numerous inflammatory mediators in bronchoalveolar lavage. Additionally, findings from gene therapy studies demonstrate that low level secretion of AAT is sufficient to induce Treg responses in the local microenvironment, favouring toleration of the associated adeno-associated virus capsid (52).

Many of the immunoregulatory functions ascribed to AAT are also regulated by vitamin D. Observational studies and clinical trials indicate that vitamin D incrementally improves airway health in established chronic respiratory disease, especially in individuals who are profoundly vitamin D deficient (2, 53, 54). The capacity of vitamin D to induce antimicrobial functions, to enhance clinical and immune responses to corticosteroids, to promote epithelial barrier integrity as well as to augment multiple pathways linked to peripheral tolerance are all proposed to underpin these clinical effects (1, 5, 55). It seems probable that vitamin D and AAT demonstrate both common and independent immunoregulatory activities that contribute to airway health, however the interplay between these mediators requires further investigation.

We propose a potential role for vitamin D induced AAT synthesis by human T cells which is discussed in the context of local immune regulation in the airways. Circulating levels of 25-hydroxyvitamin D, the accepted measure of vitamin D status,

correlated with AAT levels in the airways in a pediatric asthma cohort and matched non-asthmatic controls provides some support for this concept. However, it is highly unlikely that T cell-derived AAT alone accounts for this correlation. In our earlier publication we found no evidence that $1,25(OH)_2D3$ increased *SERPINA1* mRNA and/or AAT protein levels in monocytes, respiratory epithelial cells or primary hepatocytes (16). However, the capacity of vitamin D to control other pathways that regulate protease:antiprotease balance may also be relevant. This could include the previously reported downregulation by vitamin D of MMP-9 (21) which is known to inactivate AAT (56), that may further enhance the correlation between vitamin D and AAT levels in the lung.

The ChIP-Atlas database suggests there are no VDR binding sites within *SERPINA1* implying that indirect and/or non-binding effects of vitamin D may explain the association reported here. The current study does not define the mechanisms by which this may occur. However, links between circulating measures of vitamin D status and AAT levels reported here, in a study of type 2 diabetes patients (40), and suggested by a study in COVID-19 patients (57) support such an association.

Vitamin D has been shown to upregulate TGF-β levels both here and in independent studies, including in T cells and bronchial epithelial cells (32, 58). On the other hand, TGF-β has been shown to enhance VDR, with data showing that TGF-β1 increases VDR expression via SMAD3/4 in human granulosa-lutein cells (26). We speculate that the capacity of TGF-β to both independently increase AAT synthesis in hepatoma and bronchial epithelial cell lines (24, 25) as well as its capacity to enhance the response to vitamin D through increased expression of *VDR* and *RXRα*, as described here in T cells, might also contribute to the observed association between airway AAT and circulating vitamin D. Similar effects of TGF-β on VDR gene and protein expression have been reported elsewhere (26). These studies support co-operation between TGF-β1 and vitamin D to drive target gene expression. The interaction of vitamin D and TGF-β to induce an immunomodulatory response via increased local levels of AAT may contribute to the anti-inflammatory repertoire within the pleiotropic potential of TGF-β. Whether TGF-β acts to enhance the response to vitamin D by other cell types and/or other functions of interest remains to be investigated, but the latter at least is suggested by other studies in human T cells (29). As far as we are aware, whether vitamin D supplementation regulates local AAT levels in healthy or patient populations is still unknown but these data are awaited with interest.

PiZZ patients' hepatocytes synthesise normal levels of AAT mRNA and nascent polypeptide, but are unable to secrete signficant amounts of AAT into the circulation (15% of the normal circulating levels) and reduced levels are also evident in the airways (59). We previously demonstrated a loss of correlation of *SERPINA1* mRNA levels with an immunomodulatory readout, IL-10 gene expression, in PiZZ patient CD4+ T cells compared with those from controls (16). We attributed this to defective secretion of Z variant AAT protein from these cells. In hepatocytes the sequestration of Z AAT into retained polymeric species is related to the high levels of translation of the aggregation-prone variant. Our data presented here, despite the limitation of comparatively low patient numbers, confirm significantly impaired secretion of AAT is also observed in PiZZ T cells, that generally express far lower levels of AAT protein than the hepatocytes, relative to those from healthy donors. This could not be overcome by vitamin D in the presence or absence of TGF-β.

AAT deficiency such as that associated with the PiZZ mutation, is linked to an inherited increase in the risk of COPD and severe emphysema. This finding led to the paradigm that an imbalance between antiproteases and neutrophil-derived proteases contributes to the pathogenesis of emphysema (10). However, aberrant immune activation in AAT deficiency linked to lung disease also exists, with increasing support for a parallel role of anti-inflammatory properties of AAT that are lost in AAT deficiency. For example Baraldo et al. describe adaptive immune activation in T and B lymphocytes together with a marked increase in lymphoid follicles as a prominent feature in AAT deficiency (60). Similarly AAT-deficient individuals demonstrate increased circulating levels of IL-17A (61) and increased pro-inflammatory mediator profiles in the airways (44, 51).

Bronchial epithelial cells line the airways and are amongst the first cells to be activated by exogenous stimuli such as microbes, particulate matter air pollution, allergens, and other inflammatory stimuli. They are thought to play a pivotal role in controlling immune responses in the airways through actions on dendritic cells and other cells (62). Taken together our data support the responsiveness of lung T cell and airway epithelial cells to $1,25(OH)_2D3$ as drivers of the correlation of systemic 25(OH)D3 with lung AAT levels observed *in vivo*. HBECs express CYP27B1 and therefore are able to convert precursor vitamin D to the bioactive $1,25(OH)_2D3$ (38). Our finding in HBECs further supports this by demonstrating increased bioactive TGF-β synthesis. We therefore propose a model of T cell-epithelial cell interactions in which bronchial epithelial cells may generate bioactive $1,25(OH)_2D3$ and TGF-β the latter potentially supplemented by bioactive TGF-β production in CD4+ T cells, to promote anti-inflammatory functions in T cells (**Figure 6**). We previously proposed a T cell-epithelial cell interaction whereby $1,25(OH)_2D3$ enhances the production of soluble ST2, the decoy receptor that antagonizes IL-33 signaling in HBECs (33). These findings together suggest a physiological supportive network between epithelial and T cells of potential benefit in the control of chronic airway disease.

For simplicity the model proposed here highlights HBECs as a likely source of TGF-β. However, a wide range of cells in the airways may also contribute including fibroblasts, smooth muscle cells and inflammatory cells. Defining the most relevant celluar source of TGF-β, and this may not reflect a single cellular source, would likely require a conditional animal knockout model to address this. The precise cellular sources of TGF-β in the microenvironment are not central to the model. TGF-β (in all isoforms) is synthesized as an inactive precursor (a latency-associated protein bound to bioactive TGF-β) that is secreted into the matrix in complex with latent TGF-β binding protein. Activation e.g., mediated by integrins, stretch, and/or proteolysis is then required for the generation of the bioactive moiety (41).

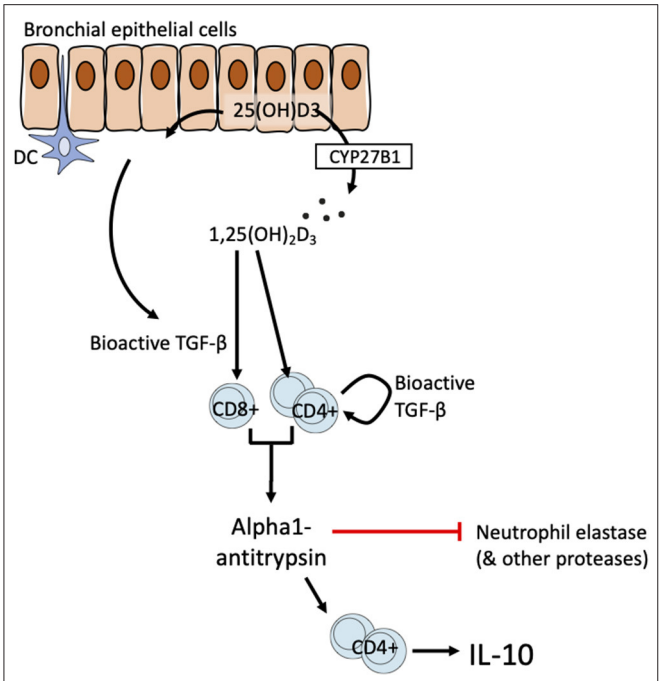

FIGURE 6 | Schematic of proposed T cell-epithelial cell interaction on the vitamin D-TGF-β-AAT axis.

Several limitations of our findings raise questions for future research. For example, whilst we demonstrate the capacity of vitamin D to increase AAT in human T cells, the lack of an obvious vitamin D response element within *SERPINA1* suggests that intermediates in this pathway and the regulation by which this effect occurs remain to be clarified. It will also be useful to define the molecular pathways involved in local generation of the active vitamin D metabolite, $1,25(OH)_2D3$, and TGF-β required for AAT induction. Whilst a correlation between circulating 25-hydroxyvitamin D and airway AAT levels in a pediatric cohort exists, it seems unlikely that this is due solely to vitamin D induction of T cell-derived AAT and the contribution of other vitamin D regulated pathways e.g., downregulation of MMP-9, remain to be fully defined. Furthermore, these data would be complemented by evidence that vitamin D supplementation of vitamin D deficient individuals increases AAT levels *in vivo*. Finally, defining complementary, distinct and/or overlapping anti-inflammatory effects of vitamin D and AAT in patients will facilitate mechanistic and future clinical studies.

CONCLUSIONS AND FUTURE RESEARCH DIRECTIONS OF THIS WORK

In summary, our study demonstrates that vitamin D increases AAT synthesis in human T cells, via a TGF-dependent mechanism, a pathway that is impaired in individuals with a genetic mutation (PiZZ) that results in decreased levels of AAT secretion. We propose that signals from adjacent airway cells, support vitamin D-mediated induction of AAT *in situ* in human lung. Some immunoregulatory properties of AAT and vitamin D overlap such as the induction of IL-10 and increase in Foxp3-Treg frequency, leading us to propose that the role of T cell-derived AAT is most likely to be immunomodulatory and may represent an intermediate of some immunodulatory functions of vitamin D. Notably, both oral vitamin D supplementation and AAT infusion are under investigation for their capacity to inhibit inflammation in a range of human immune-mediated pathologies. For AAT these data are still limited and only now emerging (49), whilst for vitamin D these data are complicated by enormous variability in part related to variable study designs (1, 4). Nevertheless, both appear to demonstrate good safety profiles and are well-tolerated, although differences in the cost of a common oral vitamin D supplement available over the counter vs. GMP-grade manufacture of AAT, are likely to be large. Comparison of biological and immune readouts of ongoing trials may provide insight into the most pertinent, overlapping and distinct effects of the two mediators. Sole AAT augmentation therapy is currently being studied, but the potential therapeutic implication of using vitamin D supplementation as an adjuvant/combination therapy to overcome any limitations faced by sole AAT therapy may be of future interest and precedent exists for the use of vitamin D as an adjunct treatment (31, 53).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Healthy donors (Human peripheral blood cell laboratory studies to investigate the role of immune and inflammatory pathways in respiratory disease by the local research ethics committee, REC reference: 14/LO/1699) were recruited and provided full written informed consent with full Research Ethics Committee approval. COPD patients without AAT deficiency (Chest clinic, Guy's Hospital, 14/LO/1699), and PiZZ-AAT deficient patients with or without COPD (Targeting dysfunctional mechanisms in alpha-1 antitrypsin deficiency, The Royal Free Alpha-1 clinic, REC reference: 13/LO/1085) were recruited and provided full written informed consent with full Research Ethics Committee approval. The study in a pediatric asthma cohort and control children undergoing diagnostic bronchoscopy for non-asthma related purposes was approved by the Royal Brompton and Harefield Research Ethics Committee (09/H07008/48) which has been previously described (14). Informed consent was obtained from parents and age-appropriate assent from children. Serum levels of 25-hydroxyvitamin D were measured as previously described (14). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

CH, BG, and Y-HC designed the study. BG recruited and screened participants. Y-HC performed functional experiments, supported by LR, SD, PP, and CC and supervised by CH and BG. SD, AG, and AB were responsible for studies in the pediatric cohort. CC, Y-HC, and CH wrote the manuscript, which all authors revised and approved. All authors contributed to the article and approved the submitted version.

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Adjunct n-3 Long-Chain Polyunsaturated Fatty Acid Treatment in Tuberculosis Reduces Inflammation and Improves Anemia of Infection More in C3HeB/FeJ Mice With Low n-3 Fatty Acid Status Than Sufficient n-3 Fatty Acid Status

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Populations at risk for tuberculosis (TB) may have a low n-3 polyunsaturated fatty acid (PUFA) status. Our research previously showed that post-infection supplementation of n-3 long-chain PUFA (LCPUFA) in TB without TB medication was beneficial in n-3 PUFA sufficient but not in low-status C3HeB/FeJ mice. In this study, we investigated the effect of n-3 LCPUFA adjunct to TB medication in TB mice with a low compared to a sufficient n-3 PUFA status. Mice were conditioned on an n-3 PUFA-deficient (n-3FAD) or n-3 PUFA-sufficient (n-3FAS) diet for 6 weeks before TB infection. Post-infection at 2 weeks, both groups were switched to an n-3 LCPUFA [eicosapentaenoic acid (EPA)/docosahexaenoic acid (DHA)] supplemented diet and euthanized at 4- and 14- days post-treatment. Iron and anemia status, bacterial loads, lung pathology, lung cytokines/chemokines, and lung lipid mediators were measured. Following 14 days of treatment, hemoglobin (Hb) was higher in the n-3FAD than the untreated n-3FAS group ($p = 0.022$), whereas the n-3FAS (drug) treated control and n-3FAS groups were not. Pro-inflammatory lung cytokines; interleukin-6 (IL-6) ($p = 0.011$), IL-1α ($p = 0.039$), MCP1 ($p = 0.003$), MIP1- α ($p = 0.043$), and RANTES ($p = 0.034$); were lower, and the anti-inflammatory cytokine IL-4 ($p = 0.002$) and growth factor GMCSF ($p = 0.007$) were higher in the n-3FAD compared with the n-3FAS mice after 14 days. These results suggest that

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n-3 LCPUFA therapy in TB-infected mice, in combination with TB medication, may improve anemia of infection more in low n-3 fatty acid status than sufficient status mice. Furthermore, the low n-3 fatty acid status TB mice supplemented with n-3 LCPUFA showed comparatively lower cytokine-mediated inflammation despite presenting with lower pro-resolving lipid mediators.

Keywords: n-3 LCPUFA, fatty acid status, adjunct therapy, C3HeB/FeJ TB model, tuberculosis, immuno-nutrition

INTRODUCTION

Tuberculosis (TB) disease is considered as an example of host immune failure (1), because of the ability of the pathogen to manipulate or evade the cellular immune responses to favor its persistence (2). TB is associated with a host inflammatory response, which results in injury to the surrounding tissue, causing significant chronic pulmonary impairment and morbidity (3–5). However, timely diagnosis and effective treatment of TB can limit infectivity and tissue damage due to the associated inflammatory effects that result in post-TB impairments, such as fibrosis and bronchiectasis, after curing (6, 7). Post-TB treatment lung impairment is a major TB-related disability burden (8). One of the common causes of this impairment is the inflammation associated with *Mycobacterium tuberculosis* (Mtb) infection (9). Thus, reducing inflammation may limit tissue damage and improve treatment, and other clinical outcomes (10). Hence, adjunctive TB therapies have been investigated frequently to augment and increase the success of standard TB treatment outcomes *via* immunomodulation (1, 11, 12). This host-directed immunotherapeutic treatment concept is designed to address the improvement of long-term outcomes and to promote a cure.

Over the past 20 years or more, the understanding of the functionality of dietary PUFAs not only as essential nutrients but their ability to also favorably modulate many disease parameters, particularly related to inflammation, has become more apparent (13). The n-3 long-chain polyunsaturated fatty acids (n-3 LCPUFAs) exert favorable health effects on several biological processes, including immune-modulation, making it a potential therapeutic agent toward combating inflammatory diseases (14–16). When supplemented in disease as a therapy, this compound has long been recognized to have anti-inflammatory activity, which has been observed in rheumatoid arthritis (17, 18), inflammatory bowel disease (19, 20), and respiratory conditions, such as asthma (21). The ability of n-3 LCPUFAs to down-regulate several mechanisms associated with inflammation, suggests that these FAs might be important in controlling the development and severity of inflammatory diseases, and they appear to be useful as components of novel therapy approaches (22), possibly also in patients with TB.

Consumption of n-3 PUFAs increases the phospholipid n-3 PUFA composition of immune cells and various tissues, thus, leading to the capacity to produce a pro-resolving lipid mediator (LM) profile upon a stimulus (23, 24). Immune cell n-3 PUFA composition is important as it influences immune functions, such as phagocytosis, neutrophil activity, and inflammatory responses (25, 26). LCPUFAs are incorporated even faster into leukocytes compared with erythrocytes, especially in an inflammatory or infectious milieu where there is rapid cell turnover (27, 28). Furthermore, pro-resolving LMs increased in plasma after 1 day of marine oil supplementation in healthy humans (29). These LMs are known as specialized pro-resolving mediators (SPMs) and include resolvins, protectins, and maresins, which contribute to inflammation resolution (14). The SPMs are known to reduce pro-inflammatory LMs, limit pro-inflammatory cytokine and chemokine production, and modify immune cell recruitment while stimulating the release of anti-inflammatory cytokines (14). An adequate presence of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in cell membranes is also known to enhance phagocytosis of apoptotic cells and bacteria (30, 31), whereas the SPMs may assist in bacterial killing (14, 32). Increased EPA and DHA intake has also been shown to inhibit a wide range of inflammatory proteins, such as tumor necrosis factor (TNF), cyclooxygenase-2 (COX-2), and interleukin-6 (IL-6) (13, 33, 34).

Prior research has shown that a good n-3 PUFA status or supplementation prior to infection is not advantageous in TB infection (35–37). However, in a recent study from our group, we demonstrated that n-3 LCPUFA supplementation administered as therapy after TB infection lowered systemic and lung inflammation in *Mtb*-infected C3HeB/FeJ mice that did not receive TB drug treatment (38). Since some populations at risk for TB has low n-3 PUFA status (39, 40), we also previously investigated the effects of n-3 PUFA supplementation on low vs. sufficient n-3 PUFA mice without TB drug treatment (41). In this earlier study without TB medication, low n-3 PUFA status mice reacted differently and showed no benefit to

Abbreviations: BSL3, Bio-safety hazard level 3; CFU, Colony-forming units; COX-2, Cyclooxygenase-2; DHA, Docosahexaenoic acid; EPA, Eicosapentaenoic acid; FA, Fatty acid; FAD, Fatty acid deficient; FAS, Fatty acid sufficient; GM-CSF, Granulocyte-macrophage colony-stimulating factor; Hb, Hemoglobin; HDHA, Hydroxydocosahexaenoic acid; HEPE, Hydroxyeicosapentaenoic acid; HETE, Hydroxyeicosatetraenoic acid; IFN-γ, Interferon-gamma; IL, Interleukin; LMs, Lipid mediators; MCP-1, Monocyte chemoattractant protein 1; MIP-1α, Macrophage inflammatory protein 1-alpha; Mtb, Mycobacterium tuberculosis; N-3 LCPUFAs, Long-chain polyunsaturated fatty acids; OADC, Oleic acid-albumin-dextrose-catalase; PD1, Protectin D1; PG, Prostaglandin; PUFA, Polyunsaturated fatty acid; PURE, Prospective Urban Rural Epidemiology; RANTES, Regulated on Activation Normal T-cell Expressed and Secreted; RH, Rifampicin and isoniazid; SPMs, Specialized pro-resolving mediators; TB, Tuberculosis; TBXB2, Thromboxane B2; TNF, Tumor necrosis factor TNF-α, Tumor necrosis factor-alpha.

supplementation after TB infection than mice with a sufficient n-3 PUFA status (35, 41).

Therefore, the current investigation focused to assess the effect of adjunct n-3 LCPUFA supplementation after infection on *Mtb*-infected C3HeB/FeJ mice with sufficient compared to low n-3 PUFA status, while being on standard TB drug treatment.

MATERIALS AND METHODS

Animals and Ethics Statement

Male and female C3HeB/FeJ mice (Jackson Laboratory, Bar Harbour, ME), aged 10 to 12 weeks, were used for the experiment. Animals were housed at biohazard level 3 (BSL3) physical containment facilities at the Faculty of Health Sciences, University of Cape Town. After infection, mice were randomly placed into groups of six in standard type 2 long individually ventilated cages with filter tops, dried wood shavings, and shredded filter paper as floor coverings. Mice were housed under a 12/12 h light/dark cycle (lights on at 06:00) at 22 ± 2°C and 55 ± 10% relative humidity, with bodyweight measured weekly. The experiments were conducted according to the South African National Guidelines (SANS 10386:2008) and the University of Cape Town practice guidelines for laboratory animal procedures. The study protocol received approval from the AnimCare Animal Research Ethics Committee of North-West University, South Africa (Ethics number: NWU-00055-19-S5), and the Animal Research Ethics Committee of the University of Cape Town, South Africa (Ethics number: FHS AEC 019-023).

Experimental Design

Figure 1 outlines the experimental design used for the study. Twenty-eight normal status 3–5 week-old pups were randomly weaned onto either an n-3 PUFA-deficient (n-3FAD) ($n = 14$) or n-3 PUFA-sufficient diet (n-3FAS) ($n = 14$) for 6 weeks, which was previously shown to produce mice with low and sufficient n-3 PUFA red blood cell (RBC) status, respectively (35, 41). Prior to infection, baseline hemoglobin (Hb) was measured. One day postinfection, four mice were euthanized to confirm the infection dose. Each group continued on their respective diets during the 2 weeks of infection, and thereafter, they switched onto an n-3 LCPUFA (EPA/DHA) supplemented diet, corresponding to the n-3FAS/n-3+ ($n = 12$) and n-3FAD/n-3+ ($n = 12$) experimental groups. Both groups also started TB antibiotics treatment at that same time. Prior to the diet switch onto the n-3 supplemented diet, pretreatment Hb was also measured to determine post infection Hb status. A total of 12 mice, 6 from the n-3FAS/n-3+ group and 6 from the n-3FAD/n-3+ group, were euthanized 4 days posttreatment in the first experimental phase (Phase 1). The remaining 12 mice, 6 of each group continued with their respective treatment protocols for an additional 10 days until euthanasia at day 14 after the initial treatment commenced, representing Phase 2 of the experimental design, as described in **Figure 1**. The body weight of the mice was measured weekly and mice had *ad libitum* access to supplemented diets and water. All mice were on standard TB antibiotics Rifafour® for 4 days of treatment (Phase 1), then rifampicin and isoniazid (RH) for the remaining 10 days (Phase 2). All anti-TB drugs used for treatment were administered either by esophageal gavage or in the drinking water. The following doses were used: in Phase 1 (intensive phase), each mouse received 0.2 ml of antibiotic consisting of Rifafour® (150 mg rifampicin + 75 mg isoniazid + 400 mg pyrazinamide + 275 mg ethambutol) dissolved in 30 ml distilled water through oral gavage administration daily. In Phase 2 (continuation phase), isoniazid (0.1 g/L) and rifampicin (0.1 g/L) were administered to mice *via* their drinking water. The water consumption was measured in Phase 2 to confirm equal drug intake in all two groups.

Experimental Diet Composition of Mice

All diets were isocaloric and contained 10% fat, with modifications of the fat source depending on whether it was a sufficient, deficient, or supplementation diet. All diets were a purified American Institute of Nutrition (AIN)-93G (42) laboratory diet formulation (commercially obtained from Dyets Inc. Bethlehem, USA). The basal AIN-93G-formulated FAS diet contained soybean oil at 70 g/kg diet and hydrogenated coconut oil at 30 g/kg diet (43). The FAD diet contained hydrogenated coconut oil at 81 g/kg diet and safflower oil at 19 g/kg diet (44–46). The n-3 LCPUFA supplementation diet fat composition contained soybean oil at 70 g/kg diet, coconut oil at 27 g/kg diet, and commercially obtained Incromega TG4030 oil (Croda Chemicals, Snaith, Europe), DHA 500 TG SR with a minimum of 44% FAs as EPA and a minimum of 28% FAs as DHA at 3 g/kg diet (38), as described in **Supplementary Table 1**. All diets were custom prepared in pellet form and stored at −20°C until use. Diets were thawed in batches, by placing them into a refrigerator (4°C). Once the bags had been opened, the diets were placed in an airtight container. The pellets were weighed weekly to determine the actual amount of food consumed. The diet was administered according to the description in **Figure 1** above.

Aerosol Infection

Virulent *Mtb* H37Rv strain was cultured and stocks were prepared and stored at −80°C, as described previously (38, 47). Mice were infected *via* aerosol by nebulizing with 6 ml of a suspension that contained 2.4×10^7 live bacteria in an inhalation exposure system (model A4224, Glas-Col) for 40 min. The mice were each infected with approximately 50–70 *Mtb* colony-forming units (CFU).

Blood and Tissue Collection

At the end of each phase of treatment, mice were euthanized by exposure to halothane, after which blood was collected *via* cardiac puncture. The blood was collected into ethylenediaminetetraacetic acid (EDTA)-coated Microtainer® tubes (K_2EDTA, 1000 µl, BD), Hb was measured in whole blood and then centrifuged at 8,000 rpm for plasma collection. The plasma was used for cytokine analyses and peripheral blood mononuclear cells (PBMCs) were collected for FA analysis. The liver, spleen, and lung lobes were removed aseptically and weighed before preparation. The left lung lobe and spleen were homogenized in saline and 0.04% Tween-80 for the analysis of the bacillary load and lung cytokines. The right superior and postcaval lung lobes and the liver were snap-frozen in

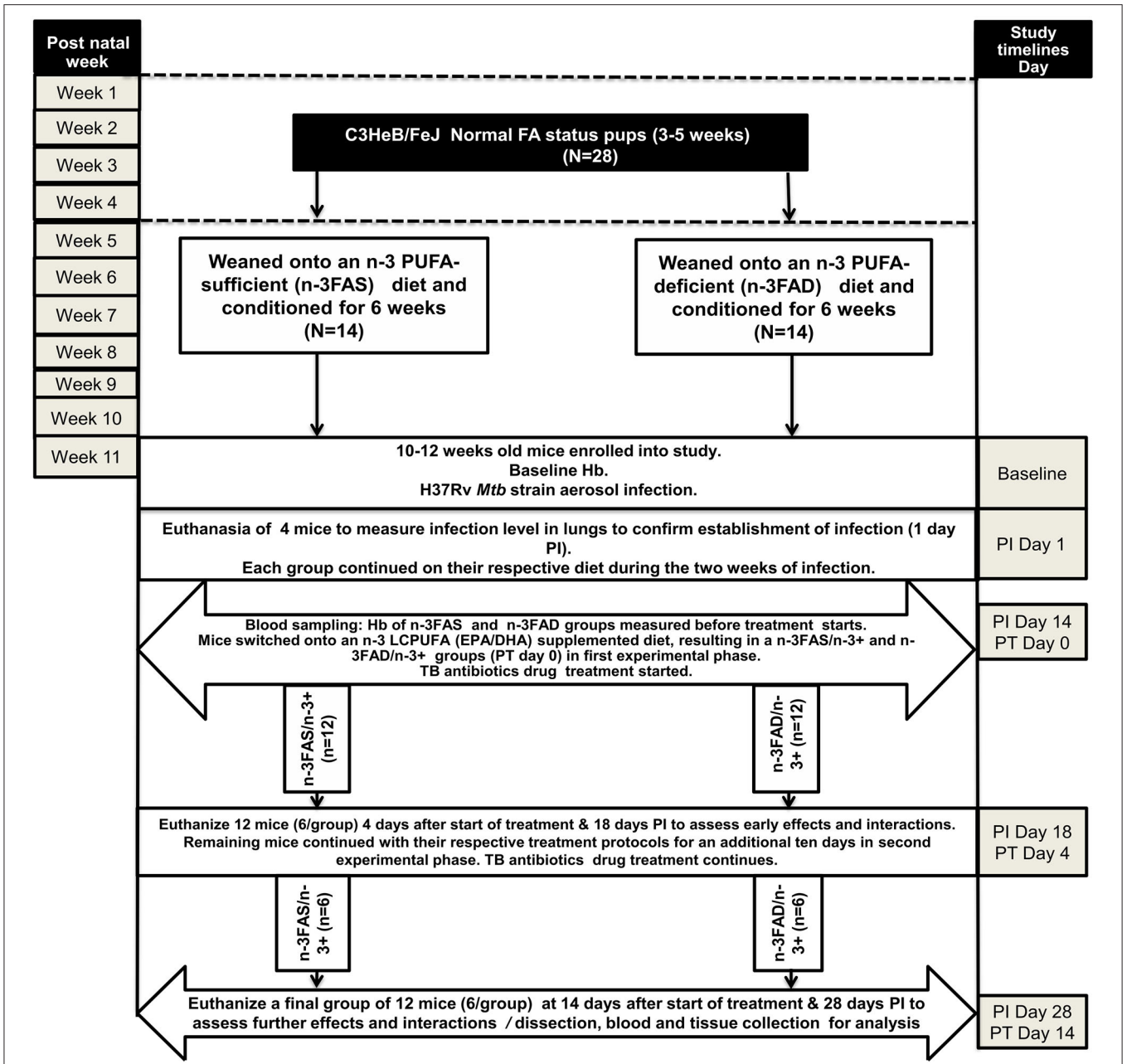

FIGURE 1 | Experimental design of the study. EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; FA: fatty acid; N: number of mice; n-PUFA: omega-3 polyunsaturated fatty acid; n-LCPUFA: omega-3 long-chain polyunsaturated fatty acid; PT, posttreatment; PI, postinfection; n-3FAS, omega-3 fatty acid-sufficient; n-3FAD, omega-3 fatty acid-deficient; n-3FAS/n-3+: omega-3 fatty acid-sufficient on EPA/DHA supplemented diet; n-3FAD/n-3+: omega-3 fatty acid-deficient on EPA/DHA supplemented diet.

liquid nitrogen and stored at −80°C for lung LM and liver iron analyses, respectively. The right middle lobe was fixed in 10% neutral buffered formalin for immunohistochemistry analysis. As a rough measure of inflammation, the lung- and spleen-weight-indexes were determined at the end of each treatment phase by taking the square root of ratios of spleen or lung tissue weight to endpoint (euthanasia) bodyweight of mice and multiplying it by 10 (47).

Hemoglobin and Liver Iron Analysis

Hemoglobin concentrations were measured in tail vein whole blood in live mice and after euthanasia blood collection in whole blood using a portable HemoCue® Hb 201+ photometer (HemoCue AB, Angelholm, Sweden). Liver iron was analyzed at the Central Analytical Facilities, Stellenbosch University, SA, on an Agilent 7900 quadrupole ICP-MS in He collision mode. The National Institute of Standards and Technology (NIST)

traceable standards were used for calibration, and the accuracy of the method was verified using the certified reference material Seronorm L2, prepared in the same way as the samples. Two n-3 FA sufficient control groups, untreated ($n = 6$) and treated with TB drugs ($n = 6$) were included in the Hb and liver iron analysis. Results for n-3 FA sufficient uninfected mice ($n = 6$) are also presented.

Immune Cell Total Phospholipid Fatty Acid Composition Analysis

The total phospholipid FA composition of peripheral blood mononuclear cell (PBMC) was analyzed by gas chromatography-tandem mass spectrometry as previously described (38). FAs were extracted from ∼200 μl PBMCs with chloroform: methanol (2:1, v:v; containing 0.01% butylated hydroxytoluene (BHT)) by a modification of the method by Folch et al. (48). Phospholipids were separated by thin-layer chromatography (TLC) (Silica gel 60 plates, Merck). The composition of EPA (20:5n-3), DHA (22:6n-3), arachidonic acid (AA, 20:4n-6), osbond acid (22:5n-6), total n-3 LCPUFA, total n-6 LCPUFA, and total n-6/n-3 LCPUFA ratio were then determined as a percentage of total phospholipid FAs. This was computed by taking the concentration of a particular FA as a percentage of the total concentration of all FAs identified in the sample.

Bacterial Load and Histopathology

The bacterial loads of the lung and spleen were determined at euthanasia, at the end of each treatment phase (4- and 14- days posttreatment). The left lobes of the lung and spleens of each mouse were removed aseptically, weighed, homogenized, and plated onto Difco™ Middlebrooks 7H10 Agar (BD Biosciences) medium with 10% oleic acid-albumin-dextrose-catalase (OADC) supplementation. The CFU was determined at 21 days following incubation at 37°C. The data are expressed as $\log_{10}$ CFU. For lung histopathology analysis, the right middle lobes of the lungs were dissected and fixed in 4% neutral buffered formalin. The fixed tissue was processed using the Leica TP 1020 Processor for 24 h and then embedded in paraffin wax. Leica sliding microtome 2000R was used to cut 2 μm thick sections of the embedded tissues. Three sections with 30 μm distance apart per tissue were obtained, deparaffinized, and stained with hematoxylin/eosin (H&E) stain. Images of the H&E slides were acquired in Nikon Eclipse 90i microscopes and analyzed with NIS-Elements AR software (Nikon Corporation, Tokyo, Japan) to ascertain the granulomatous area and free alveolar air space as a percentage of the total lung tissue as previously described (49).

Lipid Mediator Analysis

Lipid mediators in crude lung homogenates were extracted and analyzed by liquid chromatography-tandem mass spectrometry. LMs were extracted from lung tissue, in 10 μl/mg homogenization buffer (phosphate-buffered saline), with solid-phase extraction (SPE) using Strata-X (Phenomenex, Torrance, CA). The method was modified for Strata-X SPE columns from a previously described method (50). Data were quantified with Masshunter B0502, using external calibration for each compound and internal standard [PGD2-d4, PGE2-d4, PGF2-d4, and 5- and 12-HETE-d8; 1,000 pg of each (Cayman Chemicals, Ann Arbor, MI)] to correct for losses and matrix effects. Extracted and quantified LMs included: DHA-derived pro-resolving 17-hydroxydocosahexaenoic acid (17-HDHA) and protectin D1 (PD1); EPA-derived pro-resolving prostaglandin E3 (PGE3) and LM intermediates 2-, 5-, 9-, 11-, 15-, and 18-hydroxyeicosapentaenoic acid (HEPE); AA-derived pro-inflammatory intermediates 5-, 8-, 9-, 11-, 12-, and 15-hydroxyeicosatetraenoic acid (HETE); AA-derived prostaglandin D2 (PGD2), prostaglandin E2 (PGE2), and prostaglandin F2α (PGFα2); and thromboxane B2 (TBXB2).

Cytokine Analysis

Lung cell-free homogenates were prepared using the left lung lobe by homogenizing with 0.04% Tween-80 in saline. The crude homogenate was centrifuged at 3,000 *g* for 5 min, filtered, and then the supernatant was stored at −80°C until analysis. After thawing, samples were centrifuged at 6,000 rpm in a microcentrifuge for 30 min at 4°C, before mouse enzyme-linked immunosorbent assay kits were used to measure cytokines. Quansys Biosciences Q-Plex™ Mouse Cytokine Screen (West Logan, WV) Q-Plex Array 16 plex was then used to measure cytokines, which included IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12, IL-17, monocyte chemoattractant protein 1 (MCP-1), interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage inflammatory protein 1-alpha (MIP-1α), and regulated on activation normal T-cell expressed and secreted (RANTES) in the supernatant. Arrays were analyzed using the Q-View Imager Pro and Q-View Software (Quansys Biosciences Q-Plex™, West Logan, WV).

Statistical Analysis and Graphical Representation

All statistical analyses and graphics were performed with GraphPad Prism Software version 8.2 (GraphPad Software Inc., La Jolla, CA, USA). A two-sided alpha of 0.05 and a power of 80% was used to estimate a minimum sample of $n = 6$ as previously described (51). The normality of the data was evaluated by histogram visual inspection and the Kolmogorov-Smirnov test. Results are presented as means ± SE of the mean. Differences for Hb and liver iron among groups were analyzed with ANCOVA and *post-hoc* Tukey tests. Differences between the same treatments (euthanized groups) on days 4 and 14 were analyzed with independent two-tailed *t*-tests. Differences for all other markers between n-3FAD and n-3FAS groups were analyzed using independent two-tailed *t*-tests. Statistically significant differences are presented as follows: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

RESULTS

Diet Intake and Bodyweight

There were no differences between groups in the daily food intakes throughout the experiment (n-3FAS/n-3+, 3.51 ± 0.14 g; n-3FAD/n-3+, 3.74 ± 0.25). As expected, there was no significant

difference in weight gain between the groups after 4 and 14 days of n-3 LCPUFA supplementation (data not shown), indicating that differential food intake between the groups may not be an underlying reason for the observed results.

Indices of Iron Status and Anemia

There were no significant differences in the baseline (before infection) and pretreatment (before EPA/DHA supplementation) Hb levels between the n-3FAD and the n-3FAS groups (**Figures 2A,B**). **Figure 2C** shows that, between days 4 and 14 after treatment, Hb decreased in untreated n-3FAS control mice from 15.8 ± 0.5 to 14.3 ± 0.3 ($d = 0.4$, $p < 0.001$), decreased in n-3FAS mice from 15.7 ± 0.4 to 14.5 ± 0.5 ($d = 0.4$, $p = 0.001$) but tended to increase in n-3FAD mice from 13.8 ± 1.4 to 14.9 ± 0.6 ($d = 1.1$, $p = 0.07$), and had a nonsignificant increase in treated n-3FAS controls from 13.8 ± 0.8 to 14.4 ± 0.8 ($d = 0.8$, $p = 0.148$). Uninfected control mice ($n = 6$) had a Hb of 15.0 ± 0.5 (results not shown). Following 14 days of treatment, Hb was higher in the n-3FAD than the n-3FAS untreated controls ($p = 0.022$, **Figure 2C**), whereas the n-3FAS (drug) treated controls and n-3FAS group were not. These results suggest that TB-induced anemia is mitigated more by n-3 LCPUFA supplementation in the low n-3 PUFA status mice than in the sufficient status groups with and without additional n-3 LCPUFA supplementation. No significant differences were seen in the liver iron concentrations between the groups after 14 days of treatment (**Figure 2D**).

Lung Cytokines/Chemokines

Lung IL-6 ($p = 0.011$, **Figure 3A**), IL-1α ($p = 0.039$, **Figure 3B**), MCP1 ($p = 0.003$, **Figure 3C**), MIP1- α ($p = 0.043$, **Figure 3D**), and RANTES ($p = 0.034$, **Figure 3E**) were lower in the n-3FAD group when compared to the n-3FAS group, respectively, after 14 days of treatment with n-3 LCPUFA supplementation. These results suggest that a low n-3 FA status supplemented with n-3 LCPUFA as an adjunct anti-TB therapy has comparatively better inflammation-lowering effects compared with mice with a prior n-3 FA sufficient status. The reduction in pro-inflammatory cytokines showed a concomitant increase in anti-inflammatory cytokine IL-4 ($p = 0.002$, **Figure 3F**) and hematopoietic growth factor GM-CSF ($p = 0.007$, **Figure 3G**) in the n-3FAD group compared to the n-3FAS group after 14 days of treatment. This result suggests that the adjunct n-3 LCPUFA supplementation in the n-3FAD group showed a better lung anti-inflammatory response than in the n-3FAS group.

Tuberculosis-Associated Outcomes

We investigated bacterial loads, free alveolar space, and H&E stained histology sections of *Mtb*-infected mice, after 2 weeks of the dietary intervention. There were no significant differences in the lung and spleen bacteria loads between the n-3FAD and the n-3FAS supplemented groups (**Figures 4A,B**). There was a trend of less free alveolar space in the n-3FAD than the n-3FAS groups comparatively at the early stage of treatment (Phase 1) [4 days posttreatment; 42 ± 1% vs. 40 ± 1%, **Figures 4C,D** (I,II)], however, the n-3FAD group measured more free alveolar space at the latter stage of treatment (Phase 2) than the compared n-3FAS group [14 days posttreatment; 47 ± 1% vs. 45 ± 1%, **Figures 4C,D** (III,IV)] although not significantly so. This indicates that in the presence of anti-TB drugs, no difference in bacterial clearance in the FAD and FAS groups due to n-3 FA supplementation is evident.

Lung Lipid Mediators

Docosahexaenoic acid -derived pro-resolving PD1 showed no significant comparative differences between the groups, neither at 4 days nor at 14 days posttreatment; however, a trend of progressively reduced levels was seen in the 14 days posttreatment samples ($p = 0.12$; **Figure 5A**). The n-3FAS group showed comparatively higher concentrations of pro-resolving 17HDHA and PGE3 than the n-3FAD group after 14 days of treatment ($p = 0.011$, and $p = 0.005$, respectively; **Figures 5B,C**). Similarly, higher concentrations of EPA-derived pro-resolving intermediates; 9-HEPE (4 days PT, $p = 0.001$, **Figure 5D**), 11-HEPE (14 days PT, $p = 0.003$, **Figure 5E**), 12-HEPE (14 days PT, $p = 0.003$, **Figure 5F**), 15-HEPE (14 days PT, $p = 0.001$, **Figure 5G**), and 18- HEPE (14 days PT, $p = 0.044$, **Figure 5H**), were observed in the n-3FAS group when compared to the n-3FAD group. The pro-inflammatory AA-derived PGF2 and PGD2 were significantly higher in the n-3FAD group after 14 days of treatment ($p = 0.001$ and $p = 0.004$, respectively, **Supplementary Figures 1A,B**), comparatively. No significant differences were seen in the concentrations of AA-derived pro-inflammatory intermediates 5-, 8-, 9-, 11-, 12-, and 15-HETE (**Supplementary Figures 1C–H**) among the n-3FAS and the n-3FAD groups.

Effects of Treatments on PBMC Fatty Acid Composition

The phospholipid FA compositions of PBMCs after 4 and 14 days of n-3 LCPUFA supplementation are presented in **Table 1**. The n-3FAS group had comparatively higher EPA, DHA, and n-3 LCPUFA compositions than the compared n-3FAD supplemented groups (4 days PT, all $p < 0.001$ and 14 days PT, all $p < 0.001$). In addition, the composition of AA, osbond acid, total n-6 LCPUFAs, and the total n-6/n-3LCPUFA ratio were higher in the n-3FAD groups in comparison to the n-3FAS groups after 4 days ($p < 0.001$) and 14 days ($p < 0.001$) of n-3 LCPUFA supplementation.

DISCUSSION AND CONCLUSION

Our results indicate that adjunct n-3 LCPUFA supplementation in drug-treated Mtb-infected mice mitigate anemia of infection more in low n-3 FA status than sufficient status mice. This is remarkable as it was previously shown that, when not treated with TB drugs, supplementing low status n-3 FA mice with n-3 LCPUFA post-TB infection showed no benefit (35, 41). Protection against anemia during TB without making iron available for the pathogen is of utmost clinical value. Furthermore, as populations at risk for TB may have a low n-3 FA status, these results are promising to support n-3 LCPUFA as an adjunct therapy to TB drugs post-TB infection particularly in these populations. These results are in parallel with the lower pro-inflammatory cytokine/chemokine responses,

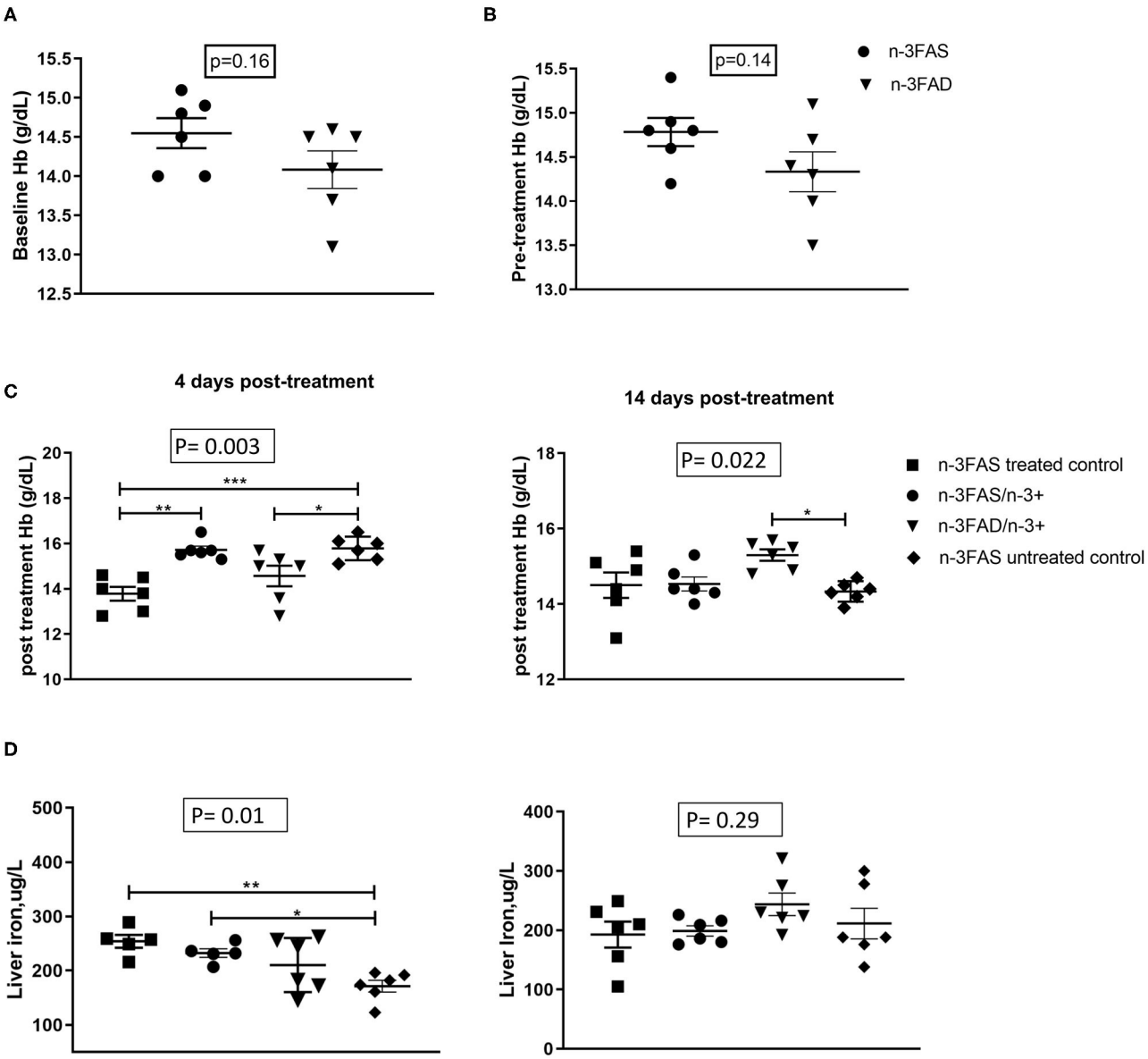

FIGURE 2 | Effect of status and n-3 long-chain polyunsaturated fatty acids (LCPUFA) supplementation on indices of anemia, **(A)** Baseline hemoglobin (Hb) levels, **(B)** Pretreatment Hb levels, **(C)** Posttreatment Hb levels, and **(D)** liver iron levels in liver homogenates. Baseline Hb levels were measured after 6 weeks of conditioning to either sufficient or low n-3 PUFA status. Pretreatment Hb levels measured after TB infection but prior to commencement of EPA/DHA supplementation. Posttreatment Hb levels were measured at each euthanasia time point after EPA/DHA supplementation. The data are represented as mean ± SEM of $n = 6$ mice/group and representative of two independent experiments. Independent *t*-tests were used to compare means, significance at $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; n-3FAS, omega-3 fatty acid- sufficient diet group; n-3FAD, omega-3 fatty acid-deficient diet group; /, switched to; n-3FAS, omega-3 fatty acid-sufficient; n-3FAS/n-3+, omega-3 fatty acid-sufficient group switched to DHA/EPA-enriched diet; n-3FAD/n-3+, omega-3 fatty acid-deficient group switched to DHA/EPA-enriched diet; n-3FAS treated control, omega-3 fatty acid-sufficient group on only tuberculosis (TB) antibiotics treatment; n-3FAS untreated control, omega-3 fatty acid-sufficient group infected with TB but not on either TB antibiotics treatment or DHA/EPA-enriched diet supplementation.

particularly IL-6, and higher anti-inflammatory IL-4 levels in the mice with previously low n-3 FA status. The aforementioned observation occurred although the low n-3 FA status mice (n-3FAD group) had comparatively less EPA, DHA, and n-3 LCPUFA compositions, and concurrent comparatively lower concentrations of EPA- and DHA-derived LMs than the sufficient status mice (n-3FAS group), after supplementation.

A study done in Brazil on patients with TB suggests that anemia could be a biomarker of TB severity and that anemia was more frequent in the most severe clinical forms of this disease, such as meningeal and disseminated TB (52). The mitigation of iron deficiency and anemia in our study is likely due to the comparatively better inflammation-lowering effect (indicated by the reduced pro-inflammatory cytokines) in the FAD group.

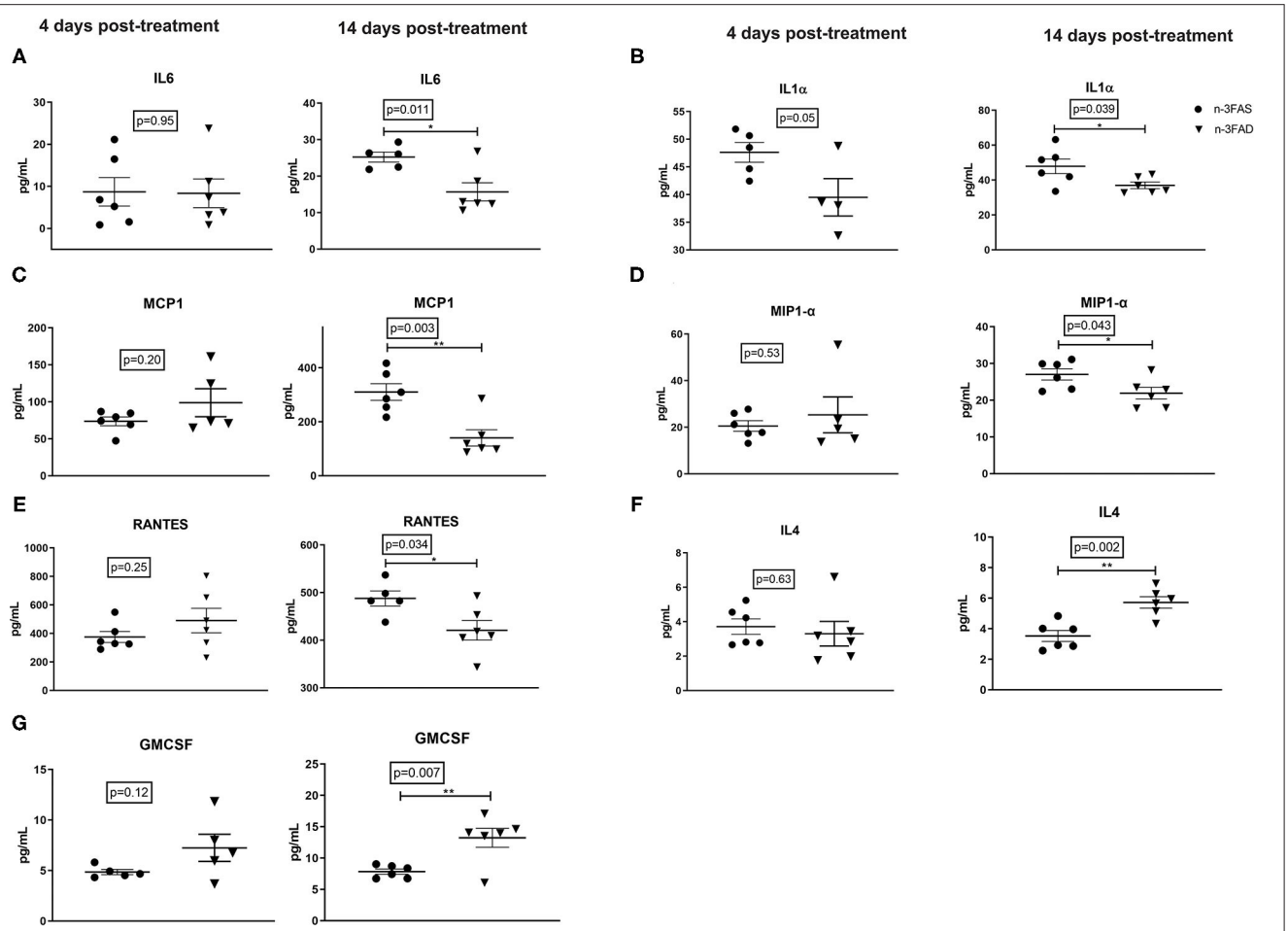

FIGURE 3 | Effects of conditioning and treatment on lung cytokine levels. **(A)** IL-6, **(B)** IL-1α, **(C)** MCP-1, **(D)** MIP-1α, **(E)** RANTES, **(F)** IL-4, and **(G)** GM-CSF. The data are represented as mean ± SEM of n = 6 mice/group and representative of two independent experiments. Unpaired two-tailed *t*-tests were used to compare means, significance at $^{*}p < 0.05$, $^{**}p < 0.01$. IL, interleukin; IL-1α, interleukin 1 alpha; MIP-1 α, macrophage inflammatory protein 1-alpha; MCP-1, Monocyte chemoattractant protein-1; GM-CSF, granulocyte-macrophage colony-stimulating factor; RANTES, Regulated on Activation, Normal T Cell Expressed and Secreted; n-3FAS, omega-3 fatty acid- sufficient diet group; n-3FAD, omega-3 fatty acid-deficient diet group.

Anemia of infection is due to a cytokine-mediated defense against microbial pathogens, which acts by effectively withholding iron from microbes, thus depriving erythroid precursors of their iron supply (53, 54). It has been demonstrated that reducing IL-6, reduces hepcidin and stops iron sequestering due to infection (55), thus mitigating iron deficiency and anemia. Omega-3 PUFA has been shown to influence iron metabolism *via* improved membrane fluidity, subsequently increasing iron uptake, improving intracellular activity (56), and improving iron stores (57).

An inflammation lowering effect in lungs of patients with TB is beneficial and a highly desirable objective during treatment, since it would improve the chances for a more favorable long-term outcome and lung health in these patients. In this study, the reduced levels of lung IL-6, IL-1α, MCP1, MIP1-α, and RANTES, seen in the FAD group when compared with the FAS group, suggest comparatively less inflammation in the initially low n-3 FA status group, when supplemented with n-3 LCPUFA. The inflammation-lowering effect observed was supported by the significantly higher concentrations of anti-inflammatory cytokine IL-4 seen in the FAD group after 14 days of treatment. The elevated levels of GM-CSF in the same FAD group is also an interesting observation since GM-CSF can increase the proliferation and phagocytic capacity of alveolar macrophages (58), necessary in granuloma formation and containment of mycobacteria (59). GM-CSF can also contribute to mycobacterial containment by polarizing macrophages into a more Mtb-restrictive phenotype (60). Combined, these findings suggest Mtb-infected mice with a low FA status, supplemented with n-3 LCPUFAs, respond better than mice with initially sufficient status. This finding is important, especially if the target intervention population is known to be at high risk of low n-3 PUFA status. In addition, considering the major economic burden associated with pulmonary TB, the use of low-cost

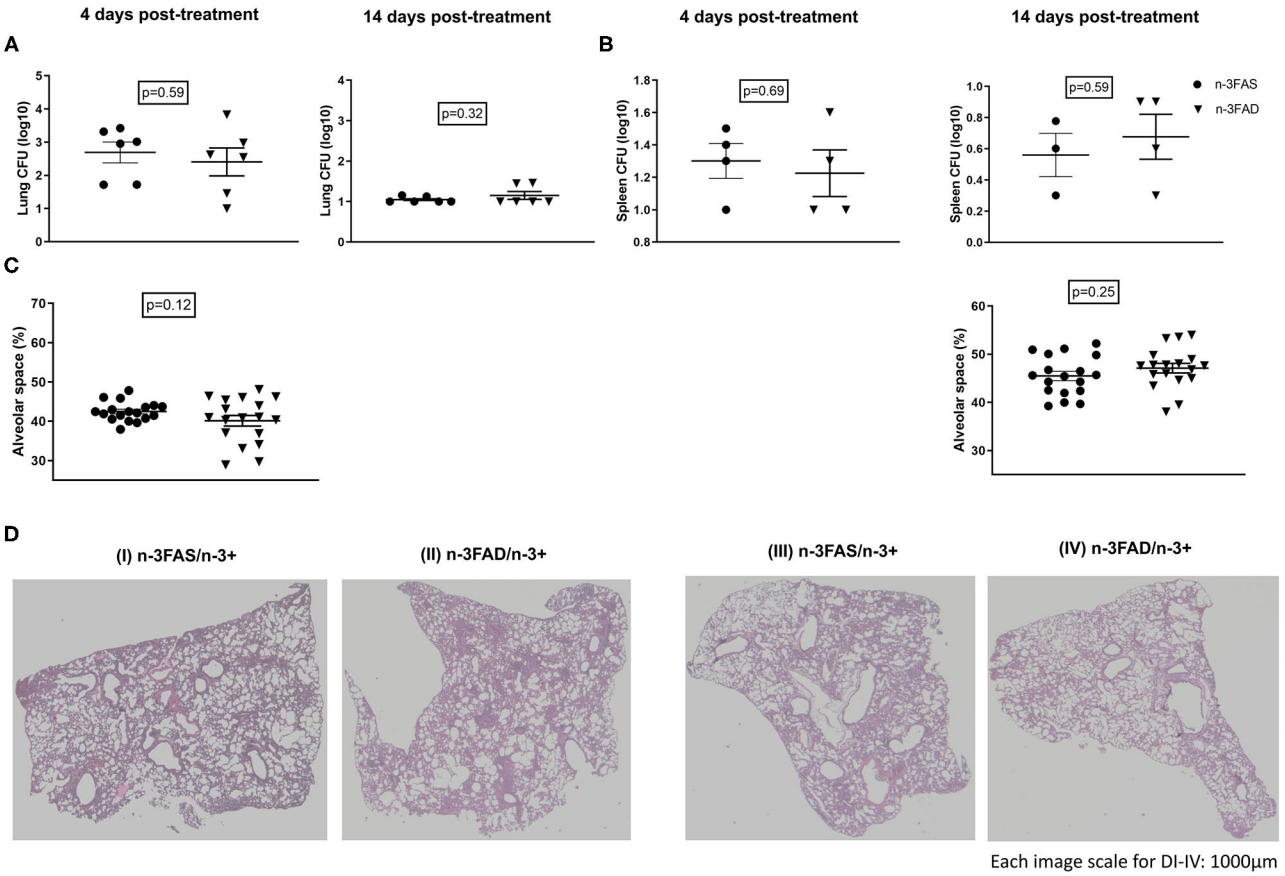

FIGURE 4 | Effect of treatments on lung bacillary load and lung histopathology according to fatty acid status. **(A)** Lung bacteria load, **(B)** spleen bacteria load, **(C)** percentage free alveolar space, and **(D)** hematoxylin–eosin-stained sections of the lungs of selected representative group, after 4 [**(D)** (I,II)] and 14 [**(D)** (III,IV)] days treatment period. The data represented as mean ± SEM of $n = 6$ mice/group and representative of two independent experiments. Unpaired two-tailed *t*-test was used to compare means, significance at $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; n-3FAS, omega-3 fatty acid-sufficient diet group; n-3FAD, omega-3 fatty acid-deficient diet group.

adjunctive therapies, such as omega-3 for such intervention, could be regarded as a cost-effective manner for improving treatment outcomes.

Despite the n-3FAS group showed comparatively a better pro-resolving LM profile (supported by their higher n-3 PUFA PBMC phospholipid composition), no reduction of its pro-inflammatory cytokine concentrations as compared to the FAD group was observed. Similar to our findings, the administration of fish oil has been shown to alter pro-resolving LMs, without significantly changing the cytokine concentrations in bronchoalveolar lavage fluid of rats (61). This could be explained by the effect of n-3 LCPUFAs supplementation on Th1/Th2 balance, and the inhibition of the Th2 type cytokine, IL 4 (62). Considering that resolving the inflammatory response in TB is a key in limiting tissue damage, an increase in pro-resolving mediators was expected to lead to resolution of inflammation and subsequent improvement in lung pathology (63, 64), also not apparent from our data. However, even though the FAD group presented with lower concentrations of the more biologically active EPA and DHA, which have been associated with improved health outcomes (16), adaptations in the low-status state may have led to a better inflammatory response in this group when supplemented. A possible explanation may be that mice with the deficiency status may be better primed for improved n-3 LCPUFA utilization due to their prior deficiency status (65).

We previously showed that EPA/DHA supplementation in mice with sufficient n-3 PUFA status did not interfere with the TB drug treatment when coadministered as an adjunct therapy (66). A similar trend was observed when mice with low n-3 PUFA status were supplemented with EPA/DHA, suggesting that the coadministration of EPA/DHA together with the currently used TB antibiotics has no observable adverse effects. Multidrug combination therapy approaches for TB treatment make it a candidate for possible drug-drug interactions (DDIs) or drug-nutrient interactions (DNIs) (67), which subsequently may result in poor TB treatment outcomes and the emergence of drug-resistant TB (68, 69). Considering the recent interest and the prospects associated with the use of repurposed drugs and pharmaconutrients as an adjunct therapy in TB (35, 70–72), this study further reinforces the prospect for the use of n-3 LCPUFA

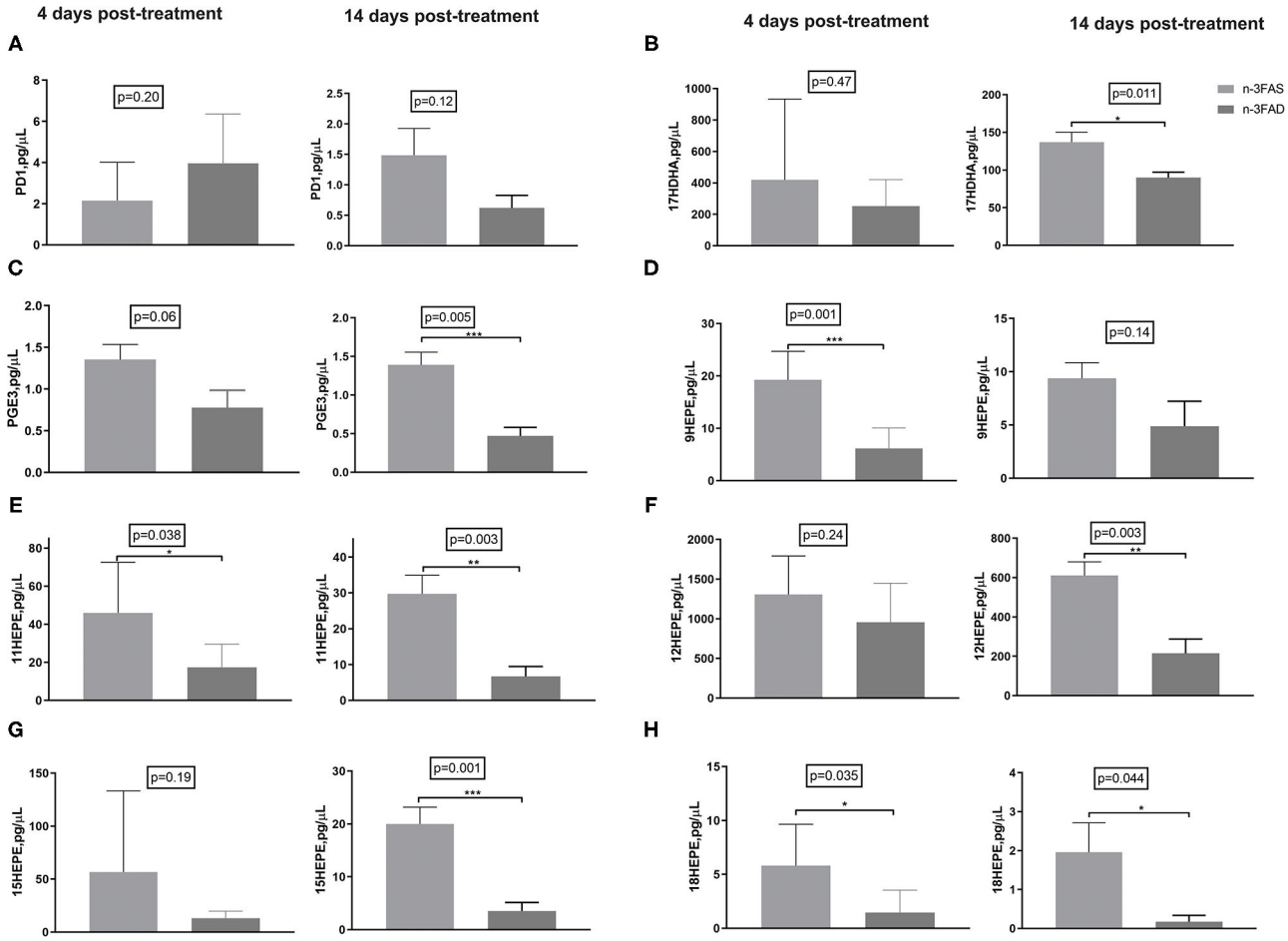

FIGURE 5 | Effects of conditioning on lipid mediators in crude lung homogenate at the local site of intervention **(A)** PD1, **(B)** 17-HDHA, **(C)** PGE3, **(D)** 9-HEPE, **(E)** 11-HEPE, **(F)** 12-HEPE, **(G)** 15-HEPE, and **(H)** 18-HEPE. The data are represented as mean ± SEM of $n = 6$ mice/group and representative of two independent experiments. Unpaired two-tailed *t*-tests were used to compare means, significance at $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. PGE3, Prostaglandin E3; PD1, Protectin D1; HDHA, hydroxydocosahexaenoic acid; HEPE, hydroxyeicosapentaenoic acid; n-3FAS, omega-3 fatty acid-sufficient diet group; n-3FAD, omega-3 fatty acid-deficient diet group.

TABLE 1 | Effects on PBMC phospholipid fatty acid composition in sufficient and low n-3 PUFA status C3HeB/FeJ mice infected with *Mtb* after receiving n-3 LCPUFA supplementation[#] ($n = 48$).

	4 days posttreatment*			14 days posttreatment**		
Fatty acids	**n-3FAS**	**n-3FAD**	***P*-value**	**n-3FAS**	**n-3FAD**	***P*-value**
20:5n-3 (EPA)	0.60 ± 0.02	0.30 ± 0.02	< 0.001	0.74 ± 0.08	0.16 ± 0.04	< 0.001
22:6n-3 (DHA)	11.54 ± 0.19	10.25 ± 0.40	<0.001	12.19 ± 0.37	9.22 ± 0.34	<0.001
Total n-3 LCPUFA	12.99 ± 0.17	10.93 ± 0.39	<0.001	14.12 ± 0.38	9.97 ± 0.38	<0.001
20:4n-6 (AA)	18.38 ± 0.38	21.08 ± 0.39	< 0.001	17.24 ± 0.24	21.52 ± 0.25	< 0.001
22:5n-6 (Osbond)	0.89 ± 0.04	2.54 ± 0.20	<0.0001	1.07 ± 0.03	3.38 ± 0.26	<0.0001
Total n-6 LCPUFA	23.22 ± 0.46	27.54 ± 0.79	<0.001	22.51 ± 0.22	29.71 ± 0.46	<0.001
Total n-6/n-3LCPUFA ratio	1.79 ± 0.03	2.55 ± 0.15	<0.001	1.60 ± 0.05	3.01 ± 0.17	<0.001

[#]*Data are reported as means ± SEM percentage of total fatty acids of n = 6 mice/group and representative of two independent experiments. Unpaired two-tailed t-tests were used to test the effects between groups. AA, Arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LCPUFA, long-chain polyunsaturated fatty acids; n, the total number of mice used in the experiment; PBMC, peripheral blood mononuclear cell; *, all groups were on standard TB antibiotics (Rifafour®); **, all groups were on standard TB antibiotics rifampicin and isoniazid (RH); n-3FAS, omega-3 fatty acid-sufficient diet group; n-3FAD, omega-3 fatty acid-deficient diet group.*

for such purposes, as was demonstrated recently by our group to lower systemic and lung inflammation in *Mtb*-infected mice (38). This result was contrary to the findings of Bonilla et al. however, who indicated that the endogenous production of n-3 PUFAs in fat-1 mice increases their susceptibility to TB. The researcher argued that the n-3 PUFAs impaired macrophage activation and diminished the antimycobacterial response in these cells from fat-1 mice (37), hence suggesting that n-3 PUFA–supplemented diets might have a detrimental effect on immunity to *Mtb*, which raises concerns about the safety of n-3 dietary supplementation in humans with TB. However, it should be noted that transgenic fat-1 mice can produce extensive amounts of n-3 PUFAs and drastically imbalance n-6/n-3 ratios. Furthermore, the timing of the supplementation is essential and should be done after infection, which was not possible with Bonilla's experiment. Based on our results, we indicate that supplementary intake of n-3 PUFAs is perhaps physiologically a better approach than using anti-inflammatory drugs to improve TB treatment outcomes.

The strengths of this study include the following: (1) this investigation took into consideration the different time points, and hence the different phases of the inflammatory and immune response; (2) this study used the same combinations of standard TB treatment is currently used in humans; (3) this study also used a murine model that reflects human pulmonary TB pathology. A possible limitation of our study was that there were no control groups that were not supplemented with adjunct n-3 LCPUFA; however, our goal in this instance was to investigate whether supplementation response in TB was dependent on n-3 PUFA status only. Considering that populations at risk for TB may have either a low or sufficient n-3 PUFA status, it was prudent to investigate the effects of both these n-3 PUFA statuses, in combination with TB medication, before proceeding to human trials.

In conclusion, when EPA/DHA was administered post-TB infection as a treatment adjunct to standard TB medication, anemia of infection was mitigated more in the low n-3 PUFA status mice than in the sufficient status mice. It also resulted in a lower production of pro-inflammatory cytokines, while increasing the anti-inflammatory cytokines in mice with low n-3 FA status. Thus, adjunct n-3 LCPUFA therapy for TB disease shows promise for improving anemia of infection and inflammation-related clinical outcomes, particularly in those with low n-3 PUFA status.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The experiments were conducted according to the South African National Guidelines (SANS 10386:2008) and the University of Cape Town practice guidelines for laboratory animal procedures. The study protocol received approval from the AnimCare Animal Research Ethics Committee of North-West University, South Africa (Ethics number: NWU-00055-19-S5) and the Animal Research Ethics Committee of the University of Cape Town, South Africa (Ethics number: FHS AEC 019-023).

AUTHOR CONTRIBUTIONS

LM headed the project. FH, LM, RD, RB, and AN conceptualized and planned the experiments. FH, LM, MO, and SP investigated and performed the experiments and contributed to the interpretation of the results. FH, LM, and MO analyzed the data. FH took the lead in writing the manuscript. RD, FB, and SP were involved in acquiring resources and funding for the experiment. All authors provided critical feedback and helped to shape the research, analysis, and manuscript, read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2021.695452/full#supplementary-material

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REVIEW
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Immunomodulatory Role of Nutrients: How Can Pulmonary Dysfunctions Improve?

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Nutrition is an important tool that can be used to modulate the immune response during infectious diseases. In addition, through diet, important substrates are acquired for the biosynthesis of regulatory molecules in the immune response, influencing the progression and treatment of chronic lung diseases, such as asthma and chronic obstructive pulmonary disease (COPD). In this way, nutrition can promote lung health status. A range of nutrients, such as vitamins (A, C, D, and E), minerals (zinc, selenium, iron, and magnesium), flavonoids and fatty acids, play important roles in reducing the risk of pulmonary chronic diseases and viral infections. Through their antioxidant and anti-inflammatory effects, nutrients are associated with better lung function and a lower risk of complications since they can decrease the harmful effects from the immune system during the inflammatory response. In addition, bioactive compounds can even contribute to epigenetic changes, including histone deacetylase (HDAC) modifications that inhibit the transcription of proinflammatory cytokines, which can contribute to the maintenance of homeostasis in the context of infections and chronic inflammatory diseases. These nutrients also play an important role in activating immune responses against pathogens, which can help the immune system during infections. Here, we provide an updated overview of the roles played by dietary factors and how they can affect respiratory health. Therefore, we will show the anti-inflammatory role of flavonoids, fatty acids, vitamins and microbiota, important for the control of chronic inflammatory diseases and allergies, in addition to the antiviral role of vitamins, flavonoids, and minerals during pulmonary viral infections, addressing the mechanisms involved in each function. These mechanisms are interesting in the discussion of perspectives associated with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection and its pulmonary complications since patients with severe disease have vitamins deficiency, especially vitamin D. In addition, researches with the use of flavonoids have been shown to decrease viral replication *in vitro*. This way, a full understanding of dietary influences can improve the lung health of patients.

Keywords: nutrients, lung health, pulmonary chronic diseases, asthma, COPD, SARS-CoV-2 infection, COVID-19

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INTRODUCTION

The lungs are fundamental organs of the respiratory system, whose main function involves extracting oxygen from the environment and making it available for aerobic respiration at the cellular level. Oxygen is used for the synthesis of ATP (adenosine triphosphate) and carbon dioxide is eliminated with other metabolic by-products (1, 2). However, in addition to their primarily respiratory functions, they are also important in other non-respiratory processes. A group of lungs cells, pulmonary neuroendocrine cells (PNECs), is responsible for the secretion of a variety of amines and peptides playing an important role in cell growth and differentiation (3). It is also an essential organ for the degradation and inactivation of chemical mediators, (4) in addition to participating in the optimization of cardiac output (3). There is also a cross-talk between intestine-lung that influences the maintenance of pulmonary mucosa homeostasis, as well as the response against pathogens and the development of inflammatory diseases (5).

The lungs are chronically exposed to various pathogenic or non-pathogenic environmental antigens. Therefore, maintaining a network of resident cells that continuously monitor the external environment and promote tolerance to innocuous particles is essential for pulmonary homeostasis. On the other hand, the deficiency in the immune response counts pathogens or intense inflammatory responses as a result of failures of mechanisms of tolerance, can generate damage to the tissue and lung function, contributing to the development of chronic inflammatory diseases for exemple chronic obstructive pulmonary disease (COPD) and asthma, and infections (6).

According to data from the World Health Organization (WHO), COPD is the third leading cause of death worldwide, causing 3.23 million deaths in 2019, with more than 80% of these deaths occurred in low- and middle-income countries (7, 8). Asthma is one of the main non-communicable diseases, affecting both children and adults. In 2019, about 262 million were affected by asthma and 461,000 people died (9). In addition to inflammatory lung diseases, lower respiratory infections continue to be the deadliest communicable disease in the world, ranked as the 4th leading cause of death. In 2019, 2.6 million individuals died (7). Citing examples of respiratory viral infections, it is estimated that annual influenza epidemics result in about 3 to 5 million cases of serious illness and about 290,000–650,000 respiratory deaths (10). In the current scenario, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection stands out, which mainly affects the lungs, in addition to other organs, and has already been responsible for more than 4.2 million deaths around the world (11). These data reinforce the importance of studies aimed at pulmonary health.

In this context, diet and nutrition are modifiable contributors to the development and progression of chronic diseases and lung infections. There is considerable evidence that indicates the importance of food intake in obstructive pulmonary diseases, such as asthma and COPD both in early life and in the development of the disease, as well as the importance of nutrition in the response against pulmonary infections (12). Macronutrients, micronutrients and bioactive components can influence homeostasis and protection of exacerbated inflammatory responses in lung tissue (13).

In this review, we discuss the interaction between nutrients and inflammatory (asthma and COPD) and infectious (viral infections) lung conditions. General physiological and immunological characteristics of the lungs were also reviewed, as well as perspectives on the possible immunomodulatory potential of some nutrients in SARS-CoV-2 infection.

LUNG GENERAL CHARACTERISTICS AND PULMONARY IMMUNOLOGY

The lungs are a pair of primary respiratory organs present in the chest cavity next to the mediastinum. They are covered by a thin double-layer serous membrane, the pleura (14). The conduction portion of the lungs begins at the trachea and extends to the terminal bronchioles, providing a pathway for movement and conditioning of the air entering the lungs (15).

The cells of the respiratory epithelium collaborate to heat, hydrate and remove incoming particles. Most of the respiratory epithelium is the ciliated pseudostratified columnar epithelium, which controls the actions of the mucociliary escalator, a primary lung defense mechanism that removes debris (16). Goblet cells are filled with mucin granules on their apical surface, and their primary function is to secrete mucin and create a protective layer of mucus (17). Basal cells, on the other hand, connect to the basal membrane and provide a fixation layer for hair and goblet cells,

Abbreviations: AP-1, activator protein 1; ATP, adenosine triphosphate; BALT, bronchial-associated lymphoid tissue; BCR, B-cell receptor; Ca^{2+}, calcium; CCL-2, C–C motif chemokine ligand 2; CCR9, C–C motif chemokine receptor 9; CD, cluster of differentiation; COPD, chronic obstructive pulmonary disease; COVID-19, coronavirus disease 2019; CRP, C-reactive protein; DAMPs, damage-associated molecular patterns; DCs, dendritic cells; DNA, deoxyribonucleic acid; EC, epicatechin; ECG, epicatechin-3-gallate; EGC, epigallocatechin; EGCG, epigallocatechin-3-gallate; FcαR, Fc alpha receptor; FOXP3, forkhead box transcription factor P3; GATA3, GATA-binding protein 3; GSH-Px, glutathione peroxidase; HATs, histone acetyltransferases; HCMV, *human cytomegalovirus*; HDAC, histone deacetylase; hTBE, tracheobronchial epithelium; IFN, interferon; IFNAR, interferon alpha and beta receptor; Ig, immunoglobulin; IKK, IκB kinase; IL, interleukin; ILCs, innate lymphoid cells; ISGs, interferon-stimulated genes; IκB, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor; JAK/STAT, janus kinase / signal transducer and activator of transcription; MAMPs, microbial-associated molecular patterns; $MgSO^{4,}$ magnesium sulfate; miRNAs, microRNA; NEBs, neuroepithelial bodies; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NK, natural killer cells; NLR, nucleotide-binding oligomerization domain OR Nod-like receptor; Nrf2, nuclear-related factor 2; PAMPs, pathogen-associated molecular patterns; PBMC, peripheral blood mononuclear cell; PGC, peroxisome proliferator-activated receptor-gamma coactivator; PGE 2, prostaglandin E 2; PNECs, pulmonary neuroendocrine cells; PRRs, pattern recognition receptors; RA, retinoic acid; RDS, respiratory distress syndrome; RIG-1, retinoic acid-inducible gene I; RNA, ribonucleic acid; RORγt, retinoic acid-related orphan receptor gamma t; ROS, oxygen species; RSV, respiratory syncytial virus; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SCFAs, short-chain fatty acids; SFB, segmented filamentous bacteria; SIRPα, signal regulatory protein α; SIRT1, sirtuin 1; TGF-β, transforming growth factor β; Th, T helper; TLRs, Toll-like receptors; TNFR, tumor necrosis factor receptor; Tregs, regulatory T cells; TREM2, triggering receptor expressed on myeloid cells 2; WHO, World Health Organization; ZIP10, zinc transporter 10; ω, omega.

in addition to interacting with lymphocytes and dendritic cells (DCs) (18).

The structural and functional unit of the respiratory system consists of the alveoli, which is the main location where gas exchange for the pulmonary vasculature occurs. In an adult individual, there are ∼300 million alveoli (19, 20).

The alveolar membrane is the largest surface area of the body in contact with the external environment and is continuously exposed to a wide variety of microbes and organic and inorganic particles. This constant exposure requires immunological mechanisms that tolerate innocuous particles that are inhaled and that immediately defend the host from microbial products or pathogens that can enter the lungs (21).

The complex interaction between airway epithelial cells and immune cells, together with chemokines and soluble proteins, shapes the outcome of host-pathogen interaction within the airway microenvironment (22). The airway epithelium restricts the growth of microorganisms because the cilia present in the epithelium move fluid, mucus, and trapped particles out of the lungs. Airway fluids also contain lysozyme, lactoferrin, and antimicrobial defensins that restrict microbial growth (21). Lung epithelial cells also express pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), that recognize microbial-associated molecular patterns (MAMPs); in addition to secreting a variety of antimicrobial products, such as defensins, complement proteins and collectins, assisting in the regulation and recruitment of immune cells (23).

Particles of 1 μm in size or smaller, which correspond to the size of bacterial and viral particles, are transported to the alveolar surface, where they interact with soluble components (for e.g., IgG, complement and surfactant) that, by means of opsonization, help phagocytosis by alveolar macrophages (24, 25).

In addition, the variety of surface receptors of alveolar macrophages allows them to sense the environment and to signal to lung stromal cells, with the aim of maintaining homeostasis or allowing the perception of changes in the inhaled environment. These signals can be activators (TLR 2, 4, 6; IL-1R, IFNγR, and TNFR), generally induced during conditions of poor nutrition, infections, use of antibiotics, pollution, smoking, or limitation of the microbiota; or suppressors (CD200R, SIRPα, mannose receptor, TREM2, IL-10R, and TGFβR) that are related to homeostasis and induced during conditions of balanced diet, minimal infections, limited antibiotic usage, or diverse microbiota (23).

Immune Cells in the Lungs

During homeostatic conditions in lung parenchyma, the immune cells are present in the following proportion: alveolar macrophages consist of ∼95% pulmonary leukocytes, with 1–4% lymphocytes and ∼1% neutrophils. In this context, alveolar macrophages are highly phagocytic and ingest a large number of inhaled inert particles that reach alveolar spaces, without triggering inflammatory responses, maintaining homeostasis (26). These cells are also capable to phagocytize apoptotic cells, preventing dead cells from releasing pro-inflammatory and toxic contents to the environment, while triggering the release of anti-inflammatory factors as a transforming growth factor β1 (TGF-β1) and prostaglandin E 2 (PGE 2) (27). In addition, the alveolar macrophages can also phagocytize pathogens initiating the immune response. Therefore, alveolar macrophages are phagocytic cell sentinels of the innate immune system in the lungs, providing a first line of defense and maintaining homeostasis through interaction with pulmonary epithelial cells (26).

The mucosa of the pulmonary conducting pathways has networks of DCs, especially myeloid DCs, which assist in immune surveillance and are capable of capturing antigens both within the intact epithelium and in the lumen of the airways (28).

Intraepithelial T cells (especially CD8) and T cells of the pulmonary lamina propria (especially CD4) are found in relatively high numbers in the mucosa and have an effector and/or memory phenotype, according to the expression of CD45RO, present in memory T cells (6). The lamina propria also contains some scattered B cells, which in addition to producing antibodies, can also contribute to the presentation of local antigens (6, 29).

Another important cell in pulmonary immunology is regulatory T (Tregs) cells. Tregs cells develop tolerogenic immune responses to innocuous antigens that find mucous surfaces. They express the forkhead box transcription factor P3 (FOXP3), which allows the regulation of the immune response through anti-inflammatory cytokines, such as interleukin (IL)-10 or TGF-β. The maintenance of immunological tolerance in the lungs by Tregs cells is achieved by modulating of $CD4^+$ T cells subsets–T helper (Th) 1, 2, or 17 cells (30, 31). It has been shown that the inadequate response of Treg cells can lead to greater susceptibility and spread of infections. The proposed mechanism includes damage to lung tissue secondary to exacerbated T-cell activity, so Tregs cells also play an important role in lung defense against pathogens (31).

It is described that individuals with COPD who presented a rapid decline in lung function, had lower frequencies of Tregs cells in the bronchoalveolar lavage compared to individuals with COPD with a non-rapid decline, suggesting that the inability to suppress the inflammatory response may lead to a rapid decline in lung function (32). It was also reported in patients with pulmonary emphysema that the peripheral capacity of these cells was normal both in patients with emphysema and in control subjects, however, secretion of IL-10 by Tregs cells from the entire lung of patients with emphysema was impaired, suggesting that the inflammatory medium affects the activation and function of Tregs cells in local tissues (33).

Another group of lung cells, important for supporting homeostasis and maintaining tissue integrity, consists of innate lymphoid cells (ILCs). ILCs show transcriptional and functional parallels with subsets of helper T cells (Th1, Th2, Th17), with the difference that ILCs do not have specific antigen receptors clonally distributed, responding to danger signals from the mucous epithelium (34).

ILC2s play a key role in maintaining the epithelial barrier of the respiratory tract, through the production of cytokines including IL-4, IL-5, IL-9, IL-13, and the epidermal growth factor-like molecule amphiregulin (34). It was demonstrated in the lung that the depletion of ILC2 negatively affected

the integrity of the epithelial barrier of the airways after infection by the influenza virus. This is because the depletion of ILC2 caused a failure to generate hyperplastic epithelial cells, leading to the deterioration of the epithelial lining (35). In the lung, IL-22 is produced especially by ILC3 and has also been shown to be involved in maintaining epithelial barrier function, mucus production, and tissue repair. Thus, ILCs contribute to barrier surveillance and epithelial protection and repair through coordinated interactions with other cells in the lung (34). On the other hand, ILCs can contribute to lung diseases by accumulating and/or altering their subsets. In patients with COPD, the signatures of IL-12 and the accumulation of ILC1s are elevated. IL-12 induces the conversion of ILC2s into interferon (IFN)-γ-producing ILC1s, thus contributing to the type 1 inflammatory lesion associated with COPD (36).

In addition to effector cells, the airway mucosa also has bronchial-associated lymphoid tissue (BALT), which comprises aggregates of lymphoid cells underlying Peyer's plaques. The presence of BALT is common in young children; however, its importance in adult humans has been questioned. It has been suggested that BALT may play a significant role in local immune homeostasis within the respiratory tract early in life, when important elements within central lymphoid structures are not fully mature (6, 37). Part of BALT role is related to the humoral immune response of the mucosa and more specifically to the production of immunoglobulin (Ig) A (IgA) (38).

IgA is the antibody isotype most present in the mucosal immune system and consists of its main defense mechanism. Provide the first line of defense in these locations against external agents without inducing a potentially harmful inflammatory response (39). Most of the IgA in the blood (about 90%) is monomeric (mIgA), while in pulmonary secretions, about half of the IgA is dimeric (dIgA), and most of it is in the form of secretory IgA (sIgA) (40).

In the infection context, sIgA helps protect the mucosal epithelium barrier through two main protective functions (41). The first, called immune exclusion, acts on the stromal epithelial portion, where IgA can form complexes with antigens. These immune complexes can be captured by phagocytic cells, absorbed into the vascular system or transported through the epithelium to the lumen. This immune elimination feature of IgA allows the maintenance of mucous tissues and protects against excess antigens that can cause infections (38). The second function is based on intracellular neutralization (41). In this case, IgA is able to prevent the assembly of the virus and neutralizes viral replication. Thus, it can interfere with the ability of antigens to adhere and penetrate the mucosa (42).

SIgA deficiency was demonstrated on the bronchial mucosa surface of ex-smokers with COPD. The deficiency was associated with latent or persistent herpesvirus infection, thickening of the submucosa and fibrotic remodeling of the airway walls (43). Associated epithelial damage also supports an inefficient first line of defense with decreased mucociliary clearance and IgA secretion (38). On the other hand, in asthma it is believed that the activation of granulocytes represents a driving force, since Fc alpha receptor (FcαR) is widely distributed in granulocytes. Thus, IgA can influence the fate of inflammatory diseases, inducing eosinophil degranulation and leading to the destruction and/or injury of the respiratory epithelium (38, 44).

The airway mucosa also contains PNECs and clusters of innervated cells called neuroepithelial bodies (NEBs), which are referred to as a set of "pulmonary neuroendocrine systems" (3, 45). These cells secrete amines, for example, serotonin, and peptides, for example, bombesin. PNECs play a role in the growth and differentiation of lung cells, while NEBs degranulate in the presence of hypoxia, acting as hypoxia-sensitive chemoreceptors (3). Immune cells and their organization in the lung are illustrated in **Figure 1**.

Although the lungs have mainly respiratory functions, there are descriptions of their role in non-respiratory processes. The lung has a very stretchable vasculature, which allows it to deal with variations in venous return, especially during postural changes, exercises and increased intravascular volume. When there is an increase in cardiac output, underperfused areas of the pulmonary vasculature are recruited to accommodate the increase in blood flow and prevent an increase in pulmonary arterial pressures (3). The pulmonary endothelium is also a source of fibrinolysin activator, which converts plasminogen to fibrinolysin. The lung, therefore, has an efficient fibrinolytic system, capable of smoothing clots in the pulmonary circulation (46).

In addition, the lungs are also an essential site for the degradation and inactivation of chemical mediators, playing a role in the biotransformation and detoxification of inhaled substances (3). The lungs express enzymes related to the metabolism of xenobiotics, the main enzymes being involved in the cytochrome P450 family and participating in oxidative metabolism, as well as in the metabolic bioactivation of many organic toxins, including pro-carcinogens (47). An important role of the lungs can therefore be to act as a buffer binding to xenobiotics, preventing an acute increase in systemic concentrations, as well as playing a role in the biotransformation of inhaled substances (47).

All these data showed the complexity of the mechanisms and interactions between immune cells, pulmonary epithelium cells and external agents (microbiota, innocuous agents, pathogens), for the maintenance of lung health and its respiratory activity.

NUTRIENT INTERFERENCE IN HOMEOSTASIS, LUNG INFECTIONS AND INFLAMMATORY LUNG DISEASES

Homeostasis can be defined as the stability of a complex system via internal mechanisms of self-regulation resilient to external disturbances (48). Considering that the lungs are chronically exposed to various pathogenic or non-pathogenic environmental antigens, maintaining a network of cells residing in the tissue that continuously monitors the external environment and instructs tolerance to innocuous inhaled particles is of paramount importance to ensure pulmonary homeostasis (6).

In this sense, diet and nutrition are becoming increasingly recognized as modifiable contributors to lung health, thus being a central parameter that governs the systemic immune system,

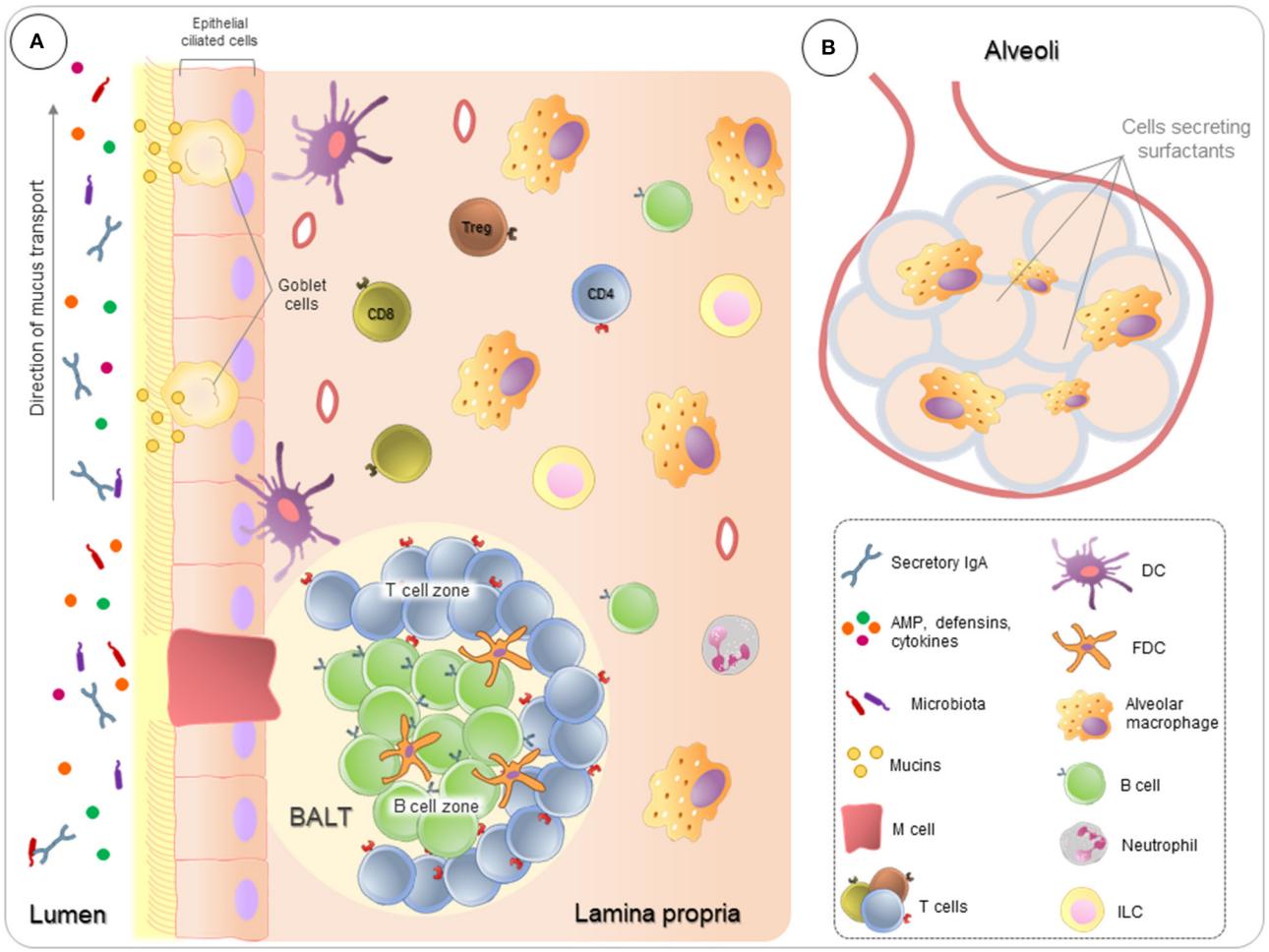

FIGURE 1 | Pulmonary immunity overview. Lungs are in constant contact with many suspended substances, which are relatively harmless, such as pollutants, microbiota, and allergens. **(A)** Ciliated pseudostratified columnar epithelium covers all respiratory tract, providing a pathway for movement and conditioning of the air entering the lungs, as well as, controls the actions of the mucociliary escalator directing the particles to outside the lung. In association, Goblet cells produce mucins to create a protective layer of mucus, forming a first barrier of defense. Airway fluids also contain antimicrobial peptides (AMP), defensins, cytokines, and antibodies, mainly secretory IgA. To ensure the homeostasis, in the lamina propria, immune cells act both to identify and respond to sterile threats and to control the inflammatory process, preventing that inflammation from compromising lung function. In addition, follicular regions rich in T and B lymphocytes (BALT–bronchus-associated lymphoid tissue) play a crucial role in fighting against infections. **(B)** Alveolus are in broadly interaction with the external environment, where lung cells secrete surfactants proteins and alveolar macrophages are highly phagocytic.

homeostasis and pulmonary inflammation. Different dietary components can have direct effects on lung health or be used as sources of energy for immune function. In fact, their resulting metabolites can be potent immune modulators (23).

However, interruption of homeostasis in response to chronic inflammation, constant levels of harmful inhalants, senescence, genetic aspects, and/or infections can lead to morphological and functional changes in the lungs (49).

COPD is a common respiratory disease characterized by functional and structural changes generated, especially, by inhaling harmful particles (49). It has been considered a disease due to the chronic inflammation leading to remodeling of the extracellular matrix, in which the extent of the inflammation is related to the degree of airflow obstruction (50). In addition to inflammation, the imbalance between proteases and antiproteases and oxidative stress are processes involved in the pathogenesis of COPD (51).

The activation of PRRs by damage-associated molecular patterns (DAMPs), which are released after tissue damage, results in the synthesis of inflammatory cytokines. One of the possible mechanisms of cytokine production involved in the pathogenesis of COPD is dependent on caspase 1 and the formation of the P3 inflammasome of the nucleotide-binding oligomerization domain (NLR) (49). The NLRP3 inflammasome leads to the secretion of IL-1β and IL-18, which activate neutrophils, macrophages, Th1 and Th17 lymphocytes, leading to inflammation of the airways (52). Neutrophils are strongly involved in inflammation, and high levels of sputum neutrophils

are associated with the severity of COPD (53). In addition, the reduced phagocytic function of macrophages influences the reduction of neutrophil apoptosis, which can generate secondary necrosis (54).

The oxidative load is also increased in COPD. The release of reactive oxygen and nitrogen species released by inflammatory cells promotes oxidative stress, which may lead to inactivation of antiproteases or stimulation of mucus production. It can also increase the activation of transcription factors (such as nuclear factor κB) and therefore, the gene expression of proinflammatory mediators (51).

Another inflammatory respiratory disease is asthma, which is a common chronic airway disorder characterized by variable and recurrent symptoms, airflow obstruction, bronchial hyperresponsiveness and underlying inflammation. Atopy, or the genetic predisposition to develop specific IgE antibodies against environmental allergens, is the strongest risk factor for the development of asthma. Although asthma has been viewed as a reversible disease, evidence indicates that permanent structural changes in the airway are typically seen and include subbasement membrane fibrosis, smooth muscle hyperplasia, new vessel formation, and glandular hyperplasia (55). In this context, long-term asthma is associated with an accelerated decline in lung function. Although lower than that observed in individuals with COPD, it is also an indication of structural and possibly permanent changes in the airways (55).

The inflammatory response in the airways of patients with asthma involves an interaction of the respiratory epithelium, innate immune system and adaptive immunity that initiates and leads to a chronic inflammatory response (56).

Prospective analyses since birth show that most asthma development occurs in early childhood and has an allergic component. Individuals with allergic asthma have eosinophilic inflammation in the lungs, as well as increased mediators related to the adaptive response of the Th2 subset, such as IL-4, IL-5 and IL-13, and serum IgE elevation. The Th17 subset may also play a role in asthma by mediating neutrophilic inflammation (57). The decrease in the suppressive activity of Treg cells (IL-10- and TGF-β-producing cells) represents additional mechanisms that contribute to asthma, perhaps partly distorting the system toward an increased Th2 response (58).

Although respiratory diseases extend throughout the course of life, the onset of asthma occurs in childhood, while COPD is commonly associated with groups in advanced age. In addition, there are significant changes in immunity associated with aging, with an increased risk of lung disease in the elderly (23).

The respiratory tract is constantly exposed to the external environment and must be prepared to respond to pathogens. Although an effective immune response to eliminate viral pathogens is essential, a prolonged or exacerbated response can cause damage to the respiratory tract. Thus, the antiviral immune response represents a balancing act between virus elimination and immune-mediated lung injury (59).

Respiratory viruses, including influenza viruses, respiratory syncytial viruses, coronaviruses, rhinoviruses, parainfluenza viruses, adenoviruses and human metapneumoviruses, among others, can lead to serious diseases in the respiratory system, such as pneumonia and/or exacerbation of asthma and chronic obstruction of lung disease (60).

Respiratory epithelial cells are usually the first cell type to be infected. The PRRs expressed in these cells recognize viral pathogen-associated molecular patterns (PAMPs) triggering the production and release of type I and III interferons and other proinflammatory mediators, such as cytokines, chemokines and antimicrobial peptides, which initiate the innate and adaptive immune response. Thus, the degree of activation of PRR influences the degree of recruitment of immune cells and release of proinflammatory mediators and, subsequently, any resulting immunopathology (59).

Type I IFNs, produced under the stimulation of viral recognition, bind to interferon alpha and beta receptor (IFNAR) expressed ubiquitously by activating the JAK/STAT pathway and inducing the expression of interferon-stimulated genes (ISGs) that act in limiting the spread and infection of the virus (61). Type I IFNs also directly promote lymphocyte functional activity by stimulating IFN-γ secretion, which in turn activates macrophages and phagocytosis, increases the presentation of antigens through DCs and limits viral replication. Type I IFNs also increase the cytotoxic activity of T cells and Natural Killer (NK) cells and promote the humoral response (62). As a consequence of widespread effects on host immune responses, IFNs can facilitate inflammation and injury to the lungs in an indirect manner during acute viral infection (59).

Diet and nutrition can be important, modifiable, risk factors for the development, progression and management of obstructive pulmonary diseases, such as asthma, COPD, and pulmonary viral infections. Dietary factors with a potential protective role in the oxidative process and inflammatory response have been implicated in the genesis, evolution or protection against these diseases (12). Below, the main nutrients that can influence homeostasis and lung diseases are described (**Figure 2**). In addition, immunomodulatory role of nutrients in these pulmonary diseases are exemplified in **Table 1**.

Vitamins

Vitamins are micronutrients available in several kinds of foods and can be of animal or vegetable origin. In addition to their nutritional role, they also participate in immunity and homeostasis of the mucosa, such as the intestinal and pulmonary mucosa. Regarding lung health and homeostasis, vitamins A, C, and D can be considered the most important, not only for their anti-inflammatory action but also for participating in the immune response against pathogens, as we describe below.

Several studies on vitamin A have shown that the active metabolite retinoic acid (RA) has a fundamental role in the maintenance and modulation of the immune response and the homeostasis of epithelial tissues and mucosa and is important in the control of inflammatory diseases (98).

In clinical studies, vitamin A deficiency can be correlated with asthma, since serum retinoid concentrations are significantly lower in patients with asthma than in healthy control subjects, mainly in patients with severe asthma (99, 100). In an experimental asthma model, vitamin A deficiency in mice also worsened the inflammatory condition by increasing the Th2

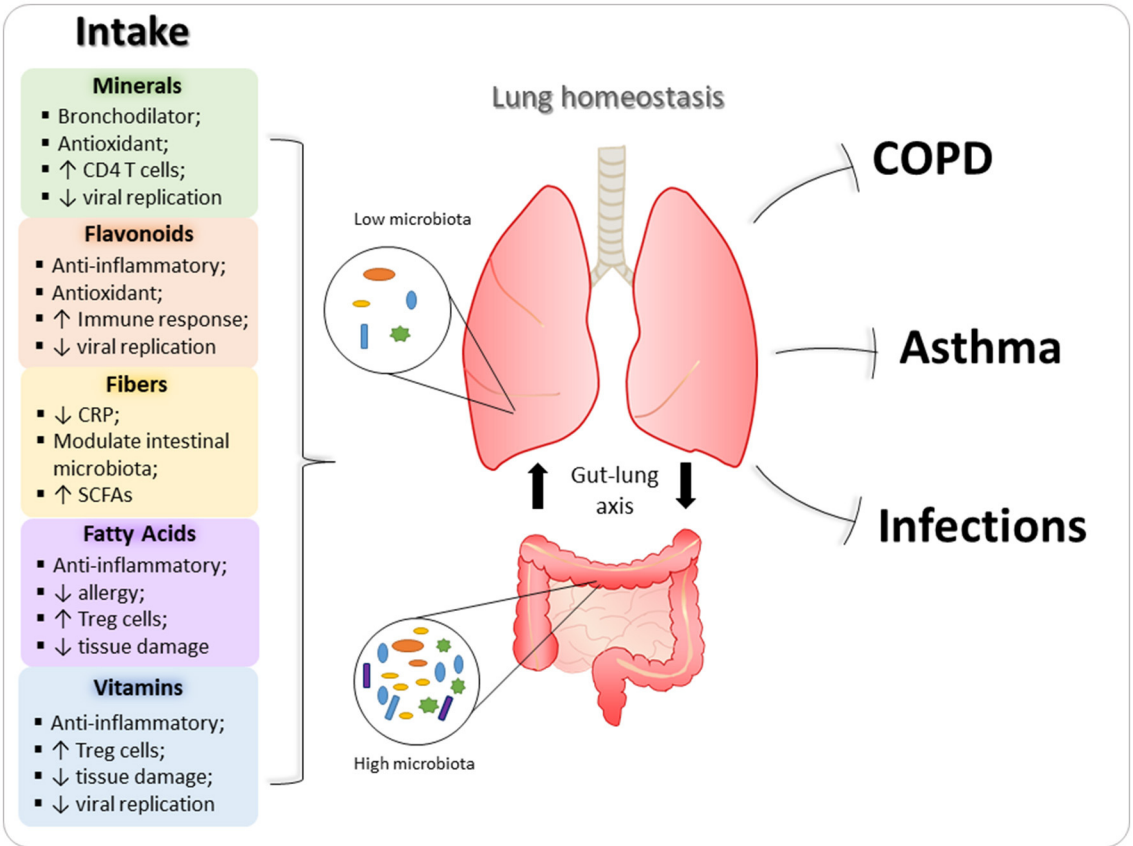

FIGURE 2 | Immunomodulatory properties of nutrients in lungs. Nutrients are able to ameliorate the development and severity of pulmonary diseases, since they can act on several immune cells and modulate immune response in inflammatory processes. In this context, minerals, flavonoids, vitamins, and fatty acids reduce the expression of inflammatory mediators (such as cytokines and chemokines), as well as, have antioxidant effect, decreasing the deleterious effects of asthma and chronic obstructive pulmonary disease (COPD) in the lungs. In addition, fibers and fatty acids also can modulate intestinal microbiota, which contribute to lung homeostasis through gut-lung axis. Regarding pulmonary viral infections, vitamins, and flavonoids are the main dietary components with antiviral action.

cytokines IL-5 and IL-13 and pulmonary inflammation (101). However, the administration of RA increases Treg cells in the lungs, attenuating inflammation (63). Supplementation with RA also promotes downregulation of the GATA3 and RORγt transcription factors, inhibiting Th2 and Th17 cytokines in the lungs (64) highlighting the importance of adequate intake of vitamin A for asthma.

Vitamin A also plays an important role in the humoral response and antiviral mechanisms of the immune response. For example, RA is essential to the production of IgA antibodies (102) and RA deficiency associated with zinc deficiency leads to a decrease in serum IgA, promoting damage to humoral immunity (103). In addition, because of vitamin A deficiency, mice infected with influenza present failures in $CD4^+$ T-cell recruitment and B-cell organization into lymphoid structures in the lungs and increasing mortality rates after bacterial coinfection (104).

Moreover, RA demonstrated the ability to decrease the viral load of *Morbillivirus* in infected mice (80). This decrease was possible due to the mechanism that induces RIG-1 expression, promoting the production of type I IFNs by increasing the recognition of viral dsRNA by immune cells. Another study showed that treatment with retinol or RA in mice infected with acute gastroenteritis virus (*Norovirus*) increased the production of IFN-β, inhibiting norovirus replication (81).

In the context of obesity, vitamin A supplementation significantly improved vitamin A levels in the lungs of diet-induced obese mice, decreased inflammatory cytokines in the blood and improved antibody responses after vaccination against the influenza A (H1N1) virus, thereby promoting a reduction in viral loads post challenge (82). These data suggest that vitamin A has a strong impact on the vaccine response.

Vitamin C is another important micronutrient for the immune response in the lungs. A trial study with 197 elderly patients with pneumonia has shown that treatment with two doses of vitamin C was able to reduce severity and mortality (105). Moreover, another antiviral effect of vitamin C could be seen in a study with H1N1-infected mice. In this study, supplementation with vitamin C increased IFN-γ production by NK cells and reduced the viral infection and lung inflammation induced by H1N1 infection (83). Similar results have been shown

TABLE 1 | Immunomodulatory proprieties of nutrients in lung inflammatory diseases.

Pathology	Immunomodulatory role of nutrients	References
Asthma	Vitamins: • Vitamin A increases Treg cells and inhibits Th2 and Th17 cytokines in the lung; • Vitamin E reduces symptoms and decrease Th2 cytokines local and systemic ways	(63–65)
	Minerals: • Magnesium has bronchodilator effects and improve lung function; • Selenium protects airways membranes from oxidative damage	(66–68)
	Flavonoids • Suppress eosinophil infiltration and inhibit degranulation of mast cells and basophils; • Decrease fibrotic factors, edema and attenuate hyperresponsiveness	(69–73)
	Fibers: • Can modify gut microbiota increasing SCFAs, which regulate neutrophils and decrease allergic process	(74)
	• Fatty acids: • ω-3 reduces asthma score, increase acetylcholine and prevented allergen-induced reactions; • SCFAs protect from allergic lung inflammation and induces Treg cells inlung	(75–77)
COPD	Vitamins: • Vitamin D deficiency is correlated with impaired control of inflammation, associated with NF-κB and AP-1 signaling activation	(78)
	Minerals: • Severe COPD is associated with lower serumzinc	(79)
	Fibers: • Reduce risk of COPD, since reduce systemic inflammations and C-reactive protein levels	(74)
Viral infections	Vitamins: • Vitamin A decreases the viral load and inflammatory cytokines, increases IFN-β, inhibits replication, and improves antibody response; • Vitamin C increases IFN-γ production by NK cells and cellular antiviral response; • Vitamin D induces antimicrobial peptides with antiviral activity and high levels of the iNF-κB inhibitor and decreases inflammatory cytokines	(80–86)
	Minerals: • Zinc induces Treg cells and the damping of Th17 and Th9 cells, increases CD4 T cells, contributes to adequate BCR antibody response, as well as inhibits RNA polymerase (necessary for the replication of RNA viruses)	(87–90)
	Flavonoids: • Improve antibody response, cytotoxic activity and increase Treg cells; • Reduces viral replication and lung injury during infection	(69, 91–95)
	Fibers: • Increases SCFAs synthesis leading to maturation and destiny of immune cells	(23)
	• Fatty acids: • SCFAs promotes recruitment of immune cells to airways and protect against infections • Acetate increases production of IFN-β and protects from respiratory infection through receptor for acetate GPR43	(74, 96, 97)

SCFAs, short-chain fatty acids; ω-3, omega-3.

in peripheral blood mononuclear cell (PBMC) culture with increases in CD25 and CD69 expression in T and NK cells, promoting the activation of the cellular antiviral response (83).

Vitamin D is important for lung health since birth, even in the absence of infections. A recent study showed a correlation between vitamin D deficiency and respiratory distress syndrome in premature infants (RDS) (106). In this same study, it was also shown that patients with higher 25(OH)D levels can be preventive for the development of RDS. RDS presents as the main characteristic of pathological surfactant deficiency and pulmonary immaturity, demonstrating the role of vitamin D in promoting lung maturity.

Vitamin D also has great potential in modulating the immune response against respiratory viruses (107). For example, vitamin D regulates the expression of antimicrobial peptides LL-37 and β-defensin 2 (108, 109), both with antiviral activity against respiratory syncytial virus (RSV) (84, 85). These antimicrobial peptides block viral cellular entry, inhibiting the production of new infectious particles and consequently, diminishing the spread of infection, and virus-induced epithelial cell death (110).

The antiviral effect of vitamin D was also described in RSV infection in the first year of life, since vitamin D deficiency has a positive correlation with RSV infection (111). In addition to antiviral effects, vitamin D also presents anti-inflammatory effects during viral infections. *In vitro* studies with primary human tracheobronchial epithelium (hTBE) pretreated with the active form of vitamin D, 1,25-dihydroxycholecalciferol [1,25(OH)2], showed less proinflammatory cytokine production after RSV infections due to high levels of the NF-κB inhibitor IκBα and IκBα induced by vitamin D pretreatment without affecting the antiviral response (86). Similar results have been shown in a treated human alveolar epithelial-cell line with

1,25(OH)2 D3 before or after influenza A (H1N1) exposure, with a decrease in proinflammatory cytokines and virus-induced cell death but without an effect on viral clearance (112).

The anti-inflammatory effect of vitamin D can also be seen in COPD patients. Recently, it has been shown that COPD patients present low serum levels of 25(OH)D and high serum levels of proinflammatory cytokines compared to healthy individuals. Vitamin D deficiency was more robust in patients with grade 4 COPD (78). These patients also presented NF-κB and activator protein 1 (AP-1) signaling activation and a decrease in the NF-κB inhibitor IκBα and IκBα. All these data showed the importance of vitamin D in controlling this inflammation.

Regarding vitamin E, there are few reports that describe its immunoregulatory role in the lungs; however, in a recent study with an experimental model of asthma and allergic rhinitis, it was demonstrated that vitamin E reduced the symptoms of the pathology due to its anti-inflammatory action (65). In this study, mice treated with vitamin E showed improvement in the pulmonary inflammatory condition, with a decrease in the presence of serum Th2 cytokines and in bronchoalveolar lavage; in addition to a decrease in constriction and mucus secretion in the mice, especially in combination with selenium.

Together, these data show the importance of adequate consumption of vitamins for the maintenance of lung health.

Minerals

Minerals are inorganic substances present in food, such as magnesium, selenium, and zinc, that play an important role in cellular metabolism and the immune response. Magnesium works with calcium (Ca^{2+}) to affect neuromuscular transmission and activity and can block or compete with calcium in voltage-dependent channels operated by receptors or leaks, resulting in translocation of intracellular Ca^{2+}. With this, magnesium inhibits the release of Ca^{2+} from the sarcoplasmic reticulum, modulating smooth-muscle contractions and the rate of relaxation (113, 114). Dietary magnesium has shown beneficial bronchodilator effects in asthma (113) as well as in severe asthma exacerbations, and a single dose of intravenous magnesium sulfate ($MgSO^4$) was able to reduce hospitalizations and improve lung function (66). On the other hand, the deficiency in magnesium consumption in asthmatic individuals was related to negative actions in the bronchial smooth muscle (12, 115).

However, it was also described in a clinical trial with asthmatic individuals that supplementation with magnesium administered together with vitamin C did not demonstrate any clinical benefit in lung function, symptoms or the possibility of decreasing the dose of steroids (116).

Selenium acts as an antioxidant and, when interacting with other nutrients, such as vitamin E, protects cells against oxidative stress. Selenium is an essential component of the enzyme glutathione peroxidase (GSH-Px), which reduces hydrogen peroxide and other organic peroxides to harmless substances. By detoxifying peroxides, GSH-Px prevents peroxidation and subsequent instability of cell membranes. It has been proposed that selenium, as a component of GSH-Px, can protect membranes in asthmatic airways from peroxide-induced damage (67).

In this context, previous studies have shown that peripheral blood and platelet GSH-Px activity is reduced in sensitive asthmatic patients (117) and case-control studies have reported lower levels of selenium in the blood of individuals with asthma compared to controls, with selenium being negatively associated with asthma (118, 119). However, a study with children did not find a relationship between the levels or intake of selenium and results related to asthma (120). Studies with adult asthmatic subjects who were on inhaled-steroid use for 24 weeks did not reveal any clinical benefit from selenium supplementation (119, 121).

Zinc is essential for the synthesis of DNA and is an enzymatic cofactor that participates in various physiological and metabolic functions in the body. It is also known to induce the production of metallothionein, which is rich in cysteine and is, therefore, a potent OH-radical scavenger (122).

In relation to control subjects, a study showed that COPD patients had lower serum zinc concentrations, and this reduction was even more pronounced in patients with COPD grade III (severe COPD) compared to those with milder disease (grades I and II) (79). Low plasma zinc content has been associated with respiratory tract infections in children, while zinc supplementation has been associated with a reduction in the incidence of pneumonia in children without vitamin A deficiency (13, 123). Zinc supplementation improves immune functions, including reduced skin hypersensitivity and an increased number of TCD4 cells (87, 124).

The altered proportion of Th1 and Th2 cells in favor of allergic reactions induced by Th2 cells is a consequence of zinc deficiency; therefore, zinc plays an important role in the proper differentiation of T cells. In addition, tolerogenic immunoreaction is triggered by changes in intracellular zinc levels due to the induction of Treg cells and the damping of proinflammatory Th17 and Th9 cells (88).

In experimental models, zinc deficiency has been shown to impair cellular and humoral immune function (125, 126), whereas, the zinc transporter 10 (ZIP10) is necessary for adequate antibody responses after B-cell receptor (BCR) activation (89).

In the context of viral infections, it has been reported that zinc is able to inhibit the RNA polymerase necessary for the replication of RNA viruses, indicating that zinc may play an essential role in the defense of the host against RNA viruses (90). The replication of influenza virus was inhibited *in vitro* by the zinc ionophore pyrrolidine dithiocarbamate (127).

In mechanistic terms, the strong correlation between homeostatic iron concentrations and the presence of oxygen in the lungs is evident, where both systems must be adequately controlled for full lung function. Oxygen to be transported efficiently by erythrocytes depends on the presence of hemoglobin, a protein capable of binding oxygen through its central iron atom (128).

In addition to participation in hemoglobin synthesis, iron is of great importance for other essential metabolic processes, such as DNA repair, transcription and energy production in mitochondria. However, free iron is highly reactive and potentially toxic and is able to catalyze the production of reactive oxygen species (ROS) and damage lipids, nucleic acids, and

proteins, causing tissue damage (129). For this reason, iron is mostly linked to protein groups to neutralize its reactivity.

Therefore, like any other cell, lung cells must acquire adequate amounts of iron to supply metabolic needs and to ensure lung function and survival. In parallel, lung cells must avoid excess iron, oxidative stress and resulting injuries that can impair lung function (130).

Flavonoids

Growing evidence suggests that natural polyphenols, particularly flavonoids, can ameliorate the inflammatory process (131). Flavonoids are polyphenolic compounds broadly present in plants (132). According to their structures and the hydroxylation and glycosylation patterns of benzene rings, flavonoids can be present in different subclasses, which include flavanols, flavanones, flavones, isoflavones, flavonols, and anthocyanidins (133). These bioactive compounds are particularly abundant in the human diet of fruits, vegetables, tea, red wine, chocolate, and coffee (134). However, their considerable structural diversity and *in vivo* bioavailability allow them to modulate different signaling pathways (135).

The immunomodulatory properties of flavonoids are associated with the inhibition of protein kinases, enzymes involved in arachidonic acid metabolism and the regulation of key signaling pathways, such as NF-κB and nuclear-related factor 2 (Nrf2) (136–140). Additionally, they have antioxidant effects due to their scavenging activity for reactive oxygen or nitrogen species and by a reduction in oxidative stress (138, 141). Some flavonoids also exert anti-inflammatory effects by blocking the NLRP3 inflammasome, inhibiting proinflammatory cytokine production, and downregulating chemokines (142–144).

Flavonoids are reported to possess a wide variety of biological activities on immune cells, modulating their activation, differentiation and proliferation, and they can act on neutrophils, T cells, NK cells, DCs, and macrophages by reducing the expression of proteins and receptors (69, 70, 138). In this context, eosinophils, neutrophils, mast cells and basophils are also affected by flavonoids, which inhibit degranulation and decrease the release of histamine and other mediators (71, 72, 145). In addition, the improvement of the immune response is associated with antibody production, cytotoxic activity, and enhancement in regulatory T cells (91).

Some studies point to the antiviral properties of flavonoids against a wide range of DNA and RNA viruses. For example, apigenin (flavone) is active against picornavirus (RNA virus), inhibiting viral activity (92); catechin (flavanol) reduces the replication cycle of the hepatitis B virus, herpes simplex and adenovirus (93); naringenin (flavanone) has antiviral activity against dengue, Zika, hepatitis C, chikungunya, yellow fever, and human immunodeficiency virus (69). In addition, flavonoids were found to reduce lung injury and the inflammatory response during influenza H1N1 infection in a mouse model (94). Recently, flavonoids have also been proposed against coronavirus infection (69, 95).

There are strong evidences that concerns the role of flavonoids in several pulmonary diseases through decreased release of inflammatory mediators, fibrotic factors, and edema, and the attenuation of Th17 inflammation and suppression of airway hyperresponsiveness (69, 72, 73). Furthermore, flavonoid supplementation is also effective in reducing the incidence of upper respiratory tract infections (146).

In general, chronic diseases are caused by chronic inflammation; therefore, flavonoids have been proposed as potentially useful treatments for inflammatory diseases.

Fibers

Dietary fibers are defined as the edible parts of plants or analogous carbohydrates. They are resistant to digestion and absorption in the human small intestine and are completely or partially fermented in the large intestine. Dietary fiber includes polysaccharides, oligosaccharides, lignin and associated plant substances. Studies indicate that fiber intake can reduce the risk of COPD due its anti-inflammatory effect, since systemic inflammation is an important feature of COPD (147).

Increased dietary fiber intake has been linked to reduced systemic inflammation and C-reactive protein (CRP) levels (74). Considering that CRP is a marker of systemic inflammation activated by the innate immune system and a possible molecule associated with vascular disease (148), it is possible that its action is related to lung damage. High-fiber diets were also able to reduce mortality from infectious or inflammatory diseases (50% reduction), respiratory diseases (60% reduction), and smoking-related cancers (25% reduction) (149).

Dietary fibers can also modify the intestinal microbiota, especially interfering with the ratio between Firmicutes and Bacteroidetes. As a result, there is an increase in short-chain fatty acids that are derived from the fermentation of dietary fibers. These fatty acids have relevant protection in the regulation of neutrophils, lung function and COPD, and epithelial protection against infection (150).

According to the European Food Safety Authority, the current recommendation for dietary fiber intake is 25 g/day (151).

Fatty Acids

Most of the lipid mediators that regulate inflammation are metabolites from omega-6 (ω-6) or omega-3 (ω-3) fatty acids, including arachidonic acid, linoleic acid, eicosapentaenoic acid and docosahexaenoic acid. ω-3 and ω-6 are considered essential fatty acids, as the body is not able to produce them, and their acquisition through diet is necessary (152). Most vegetable oils are significant sources of ω-6, while cold-water marine fish are the main sources of ω-3 (153).

Generally, ω-6 fatty acids are proinflammatory, and ω-3 fatty acids are anti-inflammatory (154). Epidemiological data describe that populations with a higher intake of ω-6 fatty acids have a higher prevalence of asthma in relation to those that consume smaller amounts of ω-6 and with a higher intake of ω-3 fatty acids, considering that ω-3 produces ecosystems that are less proinflammatory than those derived from ω-6 (154, 155).

Food supplementation with fish oil rich in ω-3 fatty acids for 10 months was able to reduce asthma scores and increase acetylcholine thresholds in children with bronchial asthma (75). In other studies, the administration of fish oil prevented only allergen-induced late asthmatic reactions and had no

effect on immediate reactions (76). In a murine model, ω-3 supplementation improved survival, reduced bacterial invasion into the blood and lungs, and decreased overall lung tissue inflammation and cell death compared to ω-6-supplemented diets (156).

Another fatty acid described in lung protection is short-chain fatty acids (SCFAs). SCFAs are derived from the fermentation of fibers by means of intestinal bacteria and are essential to regulate a wide variety of processes in the gastrointestinal tract, but they are also potent mediators of the function, maturation, and destiny of immune cells (23). Oral application of SCFAs to mice during pregnancy and weaning protected the offspring from allergic lung inflammation, potentially inducing Tregs in the offspring's lungs (77).

SCFAs such as acetate and butyrate, in addition to their anti-inflammatory activity (157), also play an important role in infectious diseases. Galvão et al. showed that the absence of the receptor for acetate GPR43 increased susceptibility to *Klebsiella pneumoniae* infection, with uncontrolled proliferation of bacteria and an inflammatory response. On the other hand, treatment with acetate was efficient for protection during bacterial lung infection (96). Against RSV infection, acetate also protects host mice through GPR43 via another mechanism. In RSV infection, the antiviral effect is caused by increasing the expression of interferon-stimulated genes in the lungs, leading to the production of IFN-β cytokine (97).

In a murine model, it was also seen that SCFAs were able to promote the recruitment of neutrophils into the airways and to protect against infection from the influenza virus (74).

GUT-LUNG AXIS AND PULMONARY IMMUNE RESPONSE

The microbiota is a constituted by the microbial commensal communities includes bacteria, fungi, viruses and protozoa that reside in different tissues, especially in gut (158). Its functions range from breaking down complex dietary polysaccharides to competing with pathogens and modulating the mucosa and the development of the immune system, both locally and systematically (159, 160). However, some studies demonstrate that the nutritional status, since childhood, impacts not only in the immune response and homeostasis but also in the intestinal microbiota, evidencing another way in which nutrition can impact mucosal immunity (161).

Regarding pulmonary health and maintenance of homeostasis, experimental evidence has highlighted a cross between the intestinal microbiota and the lungs, called the intestine-lung axis (5). Thus, the intestinal microbiota, influenced by nutrition, plays an important role in the immune responses developed during infections and inflammatory lung diseases. Below we will describe some aspects regarding the intestine-lung axis in two subsections: gut and airway microbiota.

Gut Microbiota and Gut-Lung Axis

With a bacterial load on the order of 10^{14} bacteria (162) the intestine is the most densely colonized surface of the human body, home to between 100,000 and 100 billion bacteria per ml of luminal content (163). The microorganisms present in the intestinal microbiota act as a source of PAMPs that, when recognized by PRRs as TLRs, are in direct contact with the intestinal lumen and promote the proliferation of epithelial cells, expression of antimicrobial peptides and secretion of IgA (164). In addition, the gut microbiota can influence host immunity by inducing the release of anti-inflammatory (IL-10), and proinflammatory (IFN-γ, IL-17, IL-6, and IL-12) cytokines (165), releasing metabolites (166–168), and controlling the function of phagocytes, including DCs (169).

The diversity of the intestinal microbiome has genetically determined variations, but it is also influenced by environmental factors, such as lifestyle and diet (170). For example, variations in the intake of resistant starch or non-starch polysaccharides have been reported to alter specific bacterial-rate levels, such as *Ruminococcus bromii* and *Eubacterium rectal* (171), just as the composition of the intestinal microbiota in breastfed babies (superior bifidobacteria, lactobacilli, staphylococci, and streptococci) differs considerably from formula-fed babies *(Bacteroides, Clostridia,* and *Proteobacteria)* (172). Regarding obesity, the high consumption of ultra-processed foods, in addition to causing a state of micronutrient deficiencies, may be related to dysbiosis, demonstrating the importance of diet in maintaining a healthy microbiota (173).

Dysbiosis in gut microbiota can impair immune responses and pulmonary homeostasis. In this context, studies in germ-free and antibiotic-treated mice have contributed to the understanding of the relationship between intestinal microbiota and local and systemic homeostasis (174). Fecal transplantation in these animals, thereby reconstituting their microbiota, was able to restore intestinal immunity, influence the development of the mucosal systemic immune system and protect against bacterial and viral infections (175).

An experimental model of dysbiosis induced by antibiotic ingestion decreased effector and memory T cell populations in mice infected by *Mycobacterium tuberculosis* (176), since dysbiosis affected the activation of innate receptor macrophage inducible C-type lectin (mincle) of lung DCs. After the microbiota is restored, DC's ability to activate T cells is also restored.

Dysbiosis can also impair the immune response against H1N1 infection (177), since intact microbiota composition is critical to the generation of virus-specific $CD4^+$ and $CD8^+$ T cells and antibody responses following infection in an experimental model. In addition, dysbiosis present in obesity can also be related to changes in the immune response during lung infections (173). On the other hand, promote a healthy gut microbiota is important against pulmonary infections. Study with microbiota transplantation in gut microbiota-depleted mice infected intranasally with *S. pneumoniae,* showed that microbiota was able to control bacterial dissemination and inflammation (178).

The transplantation of isolated group of host-adapted commensal organisms, such as Segmented filamentous bacteria (SFB), also play an important role in lung infections without the need for transplantation of all components of the gut microbiota.

In this context, a study with immunodeficient $Rag^{-/-}$ mice infected with *S. pneumoniae* showed that the transplantation of SFB influenced lung protection, not for controlling bacterial infection, but for regulating innate immunity (179). In this study, the SFB promoted a shift in lung neutrophil phenotype from inflammatory neutrophils to pro-resolution neutrophils with low CD18 and high CD62L reducing, this way, the severe tissue damage caused by inflammatory neutrophils. So, the gut microbiota can also act by decreasing the inflammatory response, reducing the tissue damage caused by the immune response.

Besides that, epidemiological studies have described a correlation between changes in the intestinal microbiota and susceptibility to the development of airway allergies. A reduction in the microbial variety in the intestine during childhood has been shown to increase the risk of developing asthma (180) and the use of broad-spectrum antibiotics may increase the predisposition to allergic airway diseases thus demonstrating the correlation in the intestine-lung axis.

Airway Microbiota and Gut-Lung Axis

The respiratory tract, long considered sterile, is actually a dynamic, microbial ecosystem. Unlike the intestinal microbiota, the lower respiratory tract is one of the least populated sites by microorganisms in the human body, with an approximate number of 10–100 bacteria per 1,000 cells (181). Its composition is dependent on microbial colonization of the upper respiratory tract through salivary micro-inhalations, interactions with the host's immune system and environmental conditions such as pH and oxygen concentration (182).

The intestine and lungs develop in parallel after birth, with constant communication between these two compartments (158) with the bacterial phyla most common in the lower respiratory tract being the same as those in the intestine, mainly Firmicutes and Bacteroidetes, followed by Proteobacteria and Actinobacteria (183). On the other hand, the nasal microbiota is more similar to skin microbiota, with a prevalence of Firmicutes and Actinobacteria phyla (48, 184).

The mesenteric lymphatic system is an important communication route between the intestine and the lungs, through which intact bacteria, their fragments or metabolites can translocate through the intestinal barrier, reach the circulatory system, and modulate the lung's immune response (182). For example SCFAs, which are mainly synthesized through the fermentation of bacterial dietary fibers, act in the lungs as signaling molecules in cells presenting resident antigens, thereby reducing inflammatory and allergic responses (157).

However, the gut-lung cross-talk also can influence in the opposite way, when the lung infections or chronic inflammatory diseases induce alterations in gut microbiota. Chronic lung disorders, such as asthma and COPD, can exhibit not only dysbiosis in airway microbiota but also in gut microbiota with tissue damage (185).

In addition, respiratory influenza infections in mice indirectly induce intestinal immune injury and gut dysbiosis promoting inflammation through the outgrowth of *Enterobacteriaceae* and the reduction of *Lactobacilli* and *Lactococci* (186). After gut dysbiosis mediated by IFN-γ produced by lung-$CCR9^{+}CD4^{+}$ T cells recruited into the small intestine, the population of Th17 cells increased promoting the tissue injury.

Still during mice influenza infection, changes in gut microbiota composition, reduce the acetate production and affect the bactericidal activity of alveolar macrophages (187) contributing to pulmonary pneumococcal superinfection. However, it has been shown that intranasal administration of *Lactobacillus casei* may be able to protect and mitigate the symptoms from influenza virus infection (188) in neonatal and infant mice infected. Intranasal *Bifidobacterium longum* administration also protects against viral-induced lung inflammation and injury in murine model of influenza virus infection (189). In this study, the reduced viral load was associated with reduced lung injury and IL-6 inflammatory cytokine, besides a shift from neutrophil to macrophage recruitment and increased levels of IFN-λ and surfactant protein.

The intranasal administration probiotics has also been used in inflammatory lung diseases. A study with 24 patients with chronic rhinosinusitis showed benefits with of *Lactococcus lactis W136 bacteria i*ntranasal irrigation after 14 days, with increase of the *Dolosigranulum pigrum*, a bacteria identified as potentially beneficial in the upper airways (190). Another study in a mouse model of allergic asthma reported that intranasal administration of probiotic *Lactobacillus rhamnosus GG* prevents the development of asthma due to decrease in bronchoalveolar lavage the eosinophils cells, lung IL-5 and 13 levels, and airway hyperreactivity (191).

This way, modifications in airway microbiota can contribute to protection against infections and inflammatory lung diseases.

Together, these data show the importance of gut-lung cross-talk in maintaining pulmonary mucosa homeostasis, as well as in the immune response against pathogens and the development of inflammatory diseases.

BIOACTIVE COMPOUNDS AND EPIGENETIC REGULATION IN LUNG HEALTH

Epigenetics is the transcriptional regulation of gene expression carried out by chemical changes in DNA, such as methylation, acetylation, phosphorylation, and regulation by miRNAs (microRNA), which result in phenotypic changes without promoting changes in the DNA sequence (192, 193). Transcriptional changes by acetylation are mediated by histone deacetylases (HDACs) and histone acetyltransferases (HATs). The deacetylation of histone lysine residues mediated by HDACs makes chromatin transcriptionally repressive, interfering with gene expression by inhibiting the access of transcription factors (194, 195). HAT-mediated histone acetylation makes chromatin transcriptionally permissive, thus favoring the binding of transcription factors and other transcriptional coactivators (194, 195).

In addition to histones, HDACs have other protein substrates, such as NF-κB. Sirtuin I (SIRT1), a class III HDAC, in addition to acting on histones, also acts on NF-κB, promoting deacetylation

of the p65 subunit. Therefore, SIRT1 acts to suppress the transcription of proinflammatory cytokines (196).

HDAC and HAT activity has already been identified in nuclear extracts from lung-tissue specimens. Moreover, it has been reported that patients with COPD have a progressive reduction in total HDAC activity, reflecting the severity of the disease (197). HDACs are key molecules in suppressing the production of proinflammatory cytokines; they are understood to be an important component that can act on lung health.

Bioactive compounds may play roles in the regulation of HDAC activity and histone acetylation (198). Bioactive compounds are extranutritional constituents that are usually present in food in small concentrations and provide health benefits beyond basic nutritional value (199). These bioactive molecules can have therapeutic potential by influencing energy intake, in addition to reducing the proinflammatory state, oxidative stress and metabolic disorders (200).

Epidemiological studies suggest that the increase in consumption of foods rich in bioactive compounds with antioxidant activity, such as vitamins, phytochemicals, and especially phenolic compounds, may represent an important factor in the reduction of several pathologies, such as cancer, heart disease, stroke, and Alzheimer's disease (201).

Resveratrol is a polyphenolic bioactive compound found in several plant species, including grapes and peanuts, and is able to positively regulate SIRT1 in human pulmonary alveolar epithelial cells, reduce the production of ROS and inhibit apoptosis in alveolar epithelial cells, thus reducing lung injury (202). It has also been reported that SIRT1 activates peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1α, an important regulator of mitochondrial metabolism. Therefore, resveratrol can improve mitochondrial function, which is usually compromised in the lungs of patients with COPD (203). In the context of viral infections, *in vitro* studies have indicated that treatment with a SIRT1 antagonist (EX-527) generates an increase in the production of influenza viruses and human cytomegalovirus (HCMV) infection, while SIRT1 agonists promote a reduction in the production of viral particles (204). Studies have also indicated a potential role for SIRT1 in regulating inflammation during allergic asthma due to significant inhibition of IL-6 expression (205).

However, most of the proposed therapeutic activities of resveratrol have not yet been confirmed in clinical trials. There are reports that in healthy individuals, a single dose of resveratrol (100 mg) combined with muscadine grape extract polyphenols (75 mg) is able to suppress the oxidative and inflammatory response to stress (206). There is also evidence that the safe daily dosage of resveratrol for an individual weighing 70 kg is 450 mg/day (207). However, more studies are needed.

Another bioactive compound, diferuloylmethane, is also capable of inducing epigenetic regulation and contributing to lung health. Known as curcumin or turmeric from India, this compound has a pleiotropic role, interacting with several molecular targets, such as transcription factors, proteins and enzymes associated with epigenetic modulations (208). By acting in the regulation of histone protein acetylation/deacetylation processes, it promotes changes in chromatin structure, which can influence inflammatory responses. Curcumin is able to specifically inhibit p300/CB-HAT (209).

Therefore, it promotes the suppression of histone acetylation and simultaneously promotes active activation to HDAC2 deacetylation, canceling the interaction between NF-κB and DNA; thus, preventing inflammatory responses that may be harmful to lung tissue (210). Considering that corticosteroids recruit HDAC2 as one of the mechanisms of action, it has been suggested that its induction through curcumin may be an important therapeutic target (211).

Curcumin also has direct anti-inflammatory actions through the inhibition of inhibition of IκB kinase (IKK), which degrades κB, a molecule capable of degrading the inhibitory protein of the NF-κB complex (212). In the context of viral infections, it has been reported that curcumin can provide protection against acute lung injuries induced by H1N1 infection by limiting the expansion of immune cells and reducing the production of proinflammatory cytokines via NF-κB (213).

The safe dose of curcumin is 12 grams per day; however, there are few clinical studies that have shown that ingesting curcumin can have anti-inflammatory effects, considering that one of its main disadvantages is its low bioavailability and hydrophobic nature (208).

Another compound that can influence lung health includes catechins. Catechins are among the biologically active compounds present in *Camellia sinensis*, known as green tea, and they are tea's main antioxidant agent. The catechins contained in tea include epigallocatechin-3-gallate (EGCG), epicatechin-3-gallate (ECG), epigallocatechin (EGC), and epicatechin (EC) (214). Among these, EGCG is the catechin that most demonstrates therapeutic effects. EGCG is shown to be a specific inhibitor of HAT, thus influencing histone acetylation and promoting an anti-inflammatory effect by inhibiting the P300-mediated acetylation of NK-κB. Moreover, it is also able to prevent the binding of p300. Its anti-inflammatory effect is mediated by inhibition of p300-mediated acetylation with the NK-κB promoter (208). It is described that ingestion through catechin feeding is able to improve lung health and to reduce shortness of breath and sputum in COPD (215). The average daily intake of EGCG resulting from the consumption of green tea infusions varies from 90 to 300 mg/day (216). However, further studies are needed to evaluate the most safe and effective dosage.

PERSPECTIVES ABOUT NUTRITION AND COVID-19

SARS-CoV-2 quickly spread around the world in 2020, and has been classified as a global pandemic by the World Health Organization (217). This virus is the etiologic agent of coronavirus disease 2019 (COVID-19) which presents, in general, mild, and moderate symptoms, but a more severe manifestation can cause acute respiratory syndrome, multiple organ dysfunction syndrome and can lead to death (218, 219). Indeed, a little over a year and a half after the first case of

the disease, more than 202.1 million people have already been infected with more than 4.2 million deaths (11).

Taking into account the anti-inflammatory and immunoprotective role that nutrients play in the pulmonary mucosa already discussed in the course of this review, it is not absurd to think that nutrients can be used as an important strategy against SARS-CoV-2 infection (**Figure 3**). Indeed, some studies, mostly using vitamins and antioxidant nutrients or demonstrating their deficiencies, have already shown some effect during COVID-19, as we describe below.

Perhaps the most prominent vitamin in this context is vitamin D. Many studies have already identified that there is a high incidence of vitamin D deficiency in patients with SARS-CoV-2 infection (220–223). In addition, these studies have also shown a relationship between the level of vitamin D deficiency and the severity of COVID-19. There have been many hypotheses suggested for this relationship, since the anti-inflammatory capacity of vitamin D (223) is very important in a pathology characterized by a proinflammatory cytokine storm (224) that worsens the patient's clinical condition until the possibility of affecting the need for oxygen-support therapy in patients with COVID-19 (222). This deficiency is also related to the risk of mortality from the disease (221). In an *in vitro* study with high glucose-treated monocytes, combined supplementation with vitamin D and l-cysteine was effective in lowering the risk of oxidative stress and the release of IL-8 and C–C motif chemokine ligand 2 (CCL-2) by monocytes (225). This can be relevant in patients with type 2 diabetes and COVID-19 infection, since diabetes is considered a risk factor (226). Moreover, it was recently

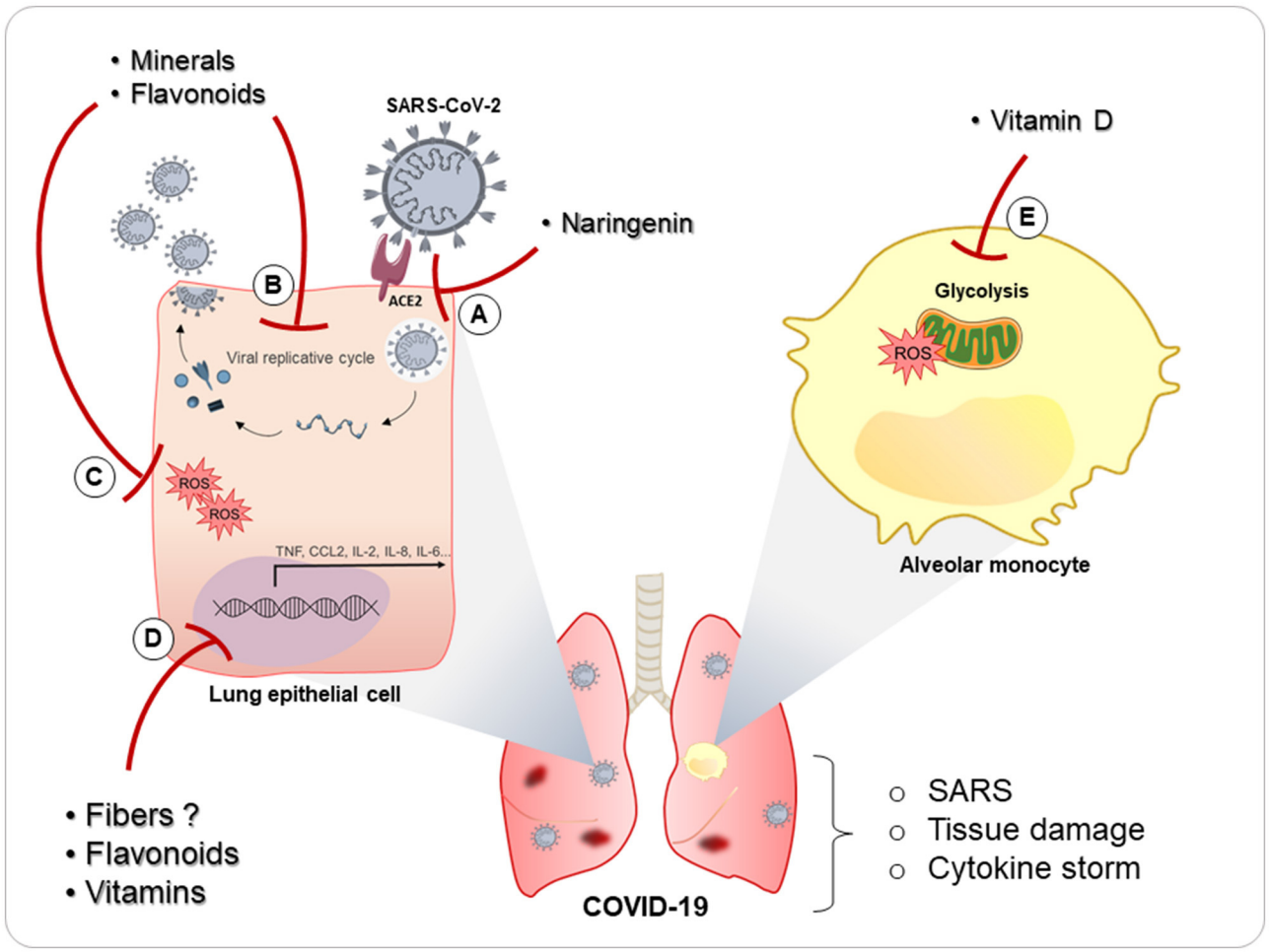

FIGURE 3 | Possible role of nutrients in COVID-19 pulmonary pathophysiology. Some nutrients have been proposed during COVID-19. (A) Naringenin has been described as being able to inhibit infection SARS-CoV-2 infection. (B) Vitamins, minerals, and flavonoids can inhibit viral replication in many pulmonary infections, and naringenin already demonstrated ability to decrease viral replication of SARS-CoV-2. (C) These nutrients also have antioxidant role (inhibition of reactive oxygen species—ROS) and (D) anti-inflammatory activity (inhibition of transcription proinflammatory factors transcription) and may inhibit the deleterious effects of the cytokine storm and tissue damage present in COVID-19. In addition, vitamin D demonstrated a relevant role on glucose-treated monocytes, lowering the risk of oxidative stress and the release of IL-8 and CCL-2 by monocytes. This can be relevant since diabetes is considered a risk factor in COVID-19 patients, in addition to the fact that monocytes glycolysis is a mechanism used by SARS-CoV-2 to promote inhibition of T cells and tissue damage in the lungs.

demonstrated that monocytes play an important role in COVID-19 pathogenicity, since SARS-CoV-2 infection triggers mitochondrial ROS production in monocytes and promotes glycolysis, inhibiting the T cell response and epithelial-cell survival in the lungs (227). Thus, the ability of vitamin D to suppress glucose-treated monocytes can be very important during SARS-CoV-2 infection.

Vitamins A, C and E have also demonstrated some importance in the prognosis of patients with COVID-19, since their deficiencies have been reported, especially in the most severe COVID-19 patients (228). Possibly, the antiviral and anti-inflammatory activity exerted by these nutrients must be impaired in those patients whose vitamin deficiency is more pronounced. In this way, vitamin supplementation in these patients is strongly suggested (229).

Another kind of nutrient deficiency in COVID-19 patients is minerals with antioxidant activity, such as selenium, zinc, magnesium, and copper (228, 230) which are essential in controlling the oxidative stress induced by SARS-CoV-2 infection.

Among flavonoids, naringenin has been shown to be a powerful inhibitor of SARS-CoV-2 infection *in vitro* (231) decreasing viral replication in Vero E6 lineage cells, demonstrating an important role in the control of viral load. In this way, naringenin can be a possible component in the treatment of patients with SARS-CoV-2 infection.

All of these data show that nutrients, in general, play an important role in the control of SARS-CoV-2 infection, which can be used as treatment strategies that may reduce the length of hospital stays and the need for respiratory support in these patients. However, more studies need to be carried out to better define the role that each nutrient may have in COVID-19 prognosis, given the vast anti-inflammatory and antiviral action that each nutrient can exert on the lung environment and the immune system in general.

CONCLUSION

The nutrients addressed in this review, in addition to their nutritional role, have a relevant role in maintaining lung health; therefore, adequate consumption of these nutrients is essential to promote an efficient immune response in the control of inflammatory diseases and infections. Moreover, the *in vitro* use of some nutrients with antiviral activity has been shown to be efficient against SARS-CoV-2 infections, which highlights the importance of these components in the current moment of the pandemic that we are facing.

AUTHOR CONTRIBUTIONS

SG-S and LO performed conception, and write and review. FT performed conception, write, and illustration and review. MS performed conception and review. AD performed review. All authors contributed to the article and approved the submitted version.

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REVIEW
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Therapeutic Potential of Nutraceuticals and Dietary Supplements in the Prevention of Viral Diseases: A Review

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Nowadays, despite enormous scientific advances, viral diseases remain the leading cause of morbidity worldwide, and their potential to spread is escalating, eventually turning into pandemics. Nutrition can play a major role in supporting the immune system of the body and for the optimal functioning of the cells of the immune system. A healthy diet encompassing vitamins, multi-nutrient supplements, functional foods, nutraceuticals, and probiotics can play a pivotal role in combating several viral invasions in addition to strengthening the immune system. This review provides comprehensive information on diet-based scientific recommendations, evidence, and worldwide case studies in light of the current pandemic and also with a particular focus on virus-induced respiratory tract infections. After reviewing the immune potential of nutraceuticals based on the lab studies and on human studies, it was concluded that bioactive compounds such as nutraceuticals, vitamins, and functional foods (honey, berries, etc.) with proven antiviral efficacy, in addition to pharmaceutical medication or alone as dietary supplements, can prove instrumental in treating a range of virus-induced infections in addition to strengthening the immune system. Milk proteins and peptides can also act as adjuvants for the design of more potent novel antiviral drugs.

Keywords: nutraceuticals, viral diseases, functional foods, immunity, coronavirus, dietary supplements

INTRODUCTION

A tremendous rise in virus-induced infections and the associated mortality rate has created the demand to come up with effective and safe antiviral drugs. Drug development in case of viruses is challenging due to the emergence of drug-resistant pathways, a limited number of targets, the rapid evolution of viral genes, and the appearance of new viral strains *via* mutations. WHO has reported around 22 different viral outbreaks in 2019 alone. From the newly emerged viral diseases like ebola virus (in the Republic of Uganda and the Democratic Republic of Congo), hantavirus (in the Republic of Panama and Argentine Republic), zika virus (in France), Middle East respiratory syndrome coronavirus (MERS-CoV) (in the Kingdom of Saudi Arabia, Oman, the United Arab Emirates, and Qatar) to well-known viral diseases like measles (in Madagascar, European Region,

Western Pacific Region, Tunisia, Lebanon, and Pacific Island Countries and Areas), and dengue fever (in Jamaica, Reunion, France, Pakistan, the Republic of Sudan, Spain, and Afghanistan), the scenario of a viral outbreak is getting worse day by day. According to WHO, the biorisk reduction is mainly based on the use of the current scientific understanding related to the viral hemorrhagic fevers, zoonotic diseases, and epidemic-prone orthopoxviruses, to develop a direction for the control, treatment, and mitigation of the risk of these viral outbreaks regardless of the source. Symptomatic treatments and immunity boost are the most efficient methods as no specific drug is available for each of the viral diseases. Even though various new antiviral agents have been developed recently, still there are numerous constraints associated with the current antiviral treatments such as efficacy, safety, and high costs (1). Thus, in this scenario, the nonconventional therapy with vitamins, multi-nutrients, functional foods, nutraceuticals, and prebiotics can play a significant role to combat this rising threat. These agents are not only virucidal in action (inhibit viral replication and protein synthesis) but can also boost the natural immunity and increase the physiological condition of the human body (like replenishing glutathione (GSH) amount and controlling the amount of free radicals in the cells). Thus, it becomes difficult for the viruses to replicate inside the host body and the severity of the symptoms also decreases, which can lead to a low mortality rate and speedy recovery (2). The natural agents (like probiotics) can directly attach to the viruses in the gut, thus preventing the latter from adhering to the host cell by various mechanisms (steric hindrance, a receptor-specific competitive/non-competitive way, the attachment of other chemical agents to prevent binding, etc.) apart from eliciting an active immune response. The antiviral activity of various natural agents against coronavirus may also be done by the modulation of the immune responses (macrophages, dendritic cells, etc.), generation of cytotoxic (antiviral) chemicals and cells like nitric oxide- (NO-) free radicals, cytotoxic T lymphocytes from CD8+ T lymphocytes, T helper cells from CD4+ T lymphocytes, activation of phagocytosis, proliferation of B lymphocytes, etc. (3).

With an increase in age, human body starts to produce a smaller number of T cells due to thymus atrophy, thus making an individual susceptible to lethal infections. Therefore, nutrition can play a significant role in assisting the immune system and in optimizing cell functions, including the cells acting in the immune function of the body. Nutraceuticals serve to functionalize food and boost the idea of diet as daily nourishment in health-related aspects. In the present review, the efficacy of nutrients, nutraceuticals, probiotics, milk proteins, and functional foods for modulating immune responses and preventing viral infections/or ameliorating disease severity has been concisely summarized. In the following sections, the use of bioactive compounds in viral mechanisms, particularly SARS and respiratory viruses-related infections, has been discussed, which might help in facilitating preintervention either directly as supplements/enriched foods or in combination with pharmaceutical medication (4).

NUTRACEUTICAL SUPPLEMENTS FOR VIRAL INFECTIONS

Nutraceuticals have antiviral, anti-inflammatory, and immunomodulatory effects, such as resveratrol (5), quercetin (6), curcumin (7), epigallocatechin gallate (EGCG) (8), N-acetyl cysteine (NAC) (9), and palmitoylethanolamide (PEA) (10) (as shown in **Table 1**). The antiviral activities of these nutraceuticals against the group of coronaviruses (like SARS-CoV-2 and COVID-19) are mainly based on their anti-inflammatory (the inhibition of NLRP3 inflammasome-mediated IL-beta production and inhibition of the pro-inflammatory cytokines) effect along with their viral replication inhibition property by regulating COVID-19 main protease (M^{pro}) (15). The viral pathogenesis is controlled, and a symptomatic relief has been provided by these agents. **Figure 1** shows a general pictorial representation of the modes of action of the selected nutraceuticals against viruses. Various nutraceuticals having an antiviral potential along with their modes of action have been discussed in the following sections.

Resveratrol

Resveratrol or 3,4′,5-trihydroxy-*trans*-stilbene is a recognized phytoalexin class of nutraceuticals (generally produced in the presence of stimuli like stress or pathogenic attack) and is a polyphenolic stilbene compound mainly found in the fermented products derived from spermatophyte family of plants such as grapes (red wine), mulberries, and peanuts. In addition to being a useful compound in the treatment of cardiovascular diseases and cancers and as a promising agent for enhancing longevity (by scavenging superoxide, hydroxyl, and lipid hydroperoxyl radicals), it has a broad spectrum of antiviral effect with proven potential *in vitro* and *in vivo*. It acts by attenuating the generation of superoxides in the mitochondria and stops arachidonic acid-induced mitochondrial dysfunction. It also inhibits virus protein production, gene expression, and nucleic acid synthesis at multiple levels (5). The antiviral properties of resveratrol have showed positive results when tested on several viruses such as influenza virus, hepatitis C virus (HCV), respiratory syncytial virus (RSV), varicella zoster virus, Epstein–Barr virus, herpes simplex virus (HSV), HIV, and African swine fever virus, and their details have been summarized in **Table 1**. However, in the case of HCV and multiple sclerosis (MS), the progression of disease worsened after RSV administration. Dose-dependent addition of resveratrol in the HCV replicon system OR6 *in vitro* significantly enhanced HCV RNA replication. Similarly, the *in vivo* study of resveratrol in mice on a viral model of MS, named autoimmune encephalomyelitis (EAE) worsened the condition of mice as compared to the control group (10, 11, 15).

Quercetin

Quercetin chemically belongs to the bioflavonoid group of nutraceuticals (flavonol), which can be widely found in fruits, vegetables, and tea. It has a broad range of actions such as signal pathway modulation, antimalignancy, antiviral, anti-inflammatory, and antioxidant. The antiviral property of quercetin possesses a wide spectrum in nature as it can be

TABLE 1 | Compilation of antiviral properties and effects of selected nutraceuticals.

Name	Virus	Mechanism of action	Effect
Resveratrol (5, 11)	Influenza virus	1. The active blocking of nucleocytoplasmic translocation of viral ribonucleoproteins in MDCK cells 2. Inhibition of protein kinase C associated mechanism.	Inhibition of *in-vitro* and *in-vivo* viral Replication and protein expression
	Epstein-Barr Virus	1. Inhibition of EBV early antigen induction (through Raji cells), EBV lytic cycle, transcription genes and proteins, Rta, Zta, and diffused early antigen (EAD), EBV immediate-early protein: BRLF1 and BZLF1 promoters, transcription factors NF-$\kappa\beta$ and AP1. 2. Downregulation of antiapoptotic proteins: Mc 1 STAT-3, miR-155, and miR-34a 3. Reduction in ROS production	1. Decrease in papilloma production, virion production 2. Inhibition of viral protein synthesis and transformation in human B-cells
	Herpes Simplex Virus	1. Decreased production of early viral protein ICP-4. 2. Induce the rapid and sustained release of ROS. 3. Inhibition of NF-$\kappa\beta$, extracellular signal-regulated kinases/mitogen-activated protein kinases (Erk/MAPK), immediate-early, early, and late HSV genes.	1. Reversible, dose-dependent inhibition of virus replication *In-vitro* and *in-vivo* 2. Prevention of viral reactivation in neuron cells, cutaneous lesions in abraded skin and vaginal lesions,
	Respiratory Syncytial Virus	1. Modulation of toll-like receptor 3 expression 2. Inhibition of toll/IL-1R domain-containing adaptor inducing IFN (TRIF) signaling, matrix metalloproteinase 12 (MMP-12), TANK binding kinase 1 (TBK1) protein expression, TNF-α, IL-2, IL-6, and nerve growth factor (NGF) secretion 3. Induction of muscarinic 2 receptor (M2R) and upregulation of sterile-α- and armadillo motif-containing protein (SARM) expression	1. Reduction in the level of interferon-gamma (IFN-γ) 2. Decreased number of inflammatory cells, reduction of inflammation reflex and airway inflammation. 3. Inhibition of viral replication
	Human Immunodeficiency Virus (HIV)	1. Inhibition DNA synthesis during the reverse transcription process 2. Activation of lytic cycle of HIV-1 *in vitro*;	1. Inhibition of HIV-1 replication *in-vitro* 2. attenuation of the Tat-induced HIV-1 LTR trans activation *in-vitro*
	Varicella Zoster Virus (VZV)	1. Reversible, dose-dependent inhibition of MRC-5 cells 2. Decrease the synthesis of intermediate early protein (IE 62)	Inhibition of VZV replication *in vitro*
	Enterovirus 71	1. Phosphorylation of proinflammatory cytokines (IKKα, IKKβ, IKKγ, IKBα, NF-$\kappa\beta$ p50, and NF-$\kappa\beta$ p65) 2. Inhibition of IL-6 and TNF-α Secretion	Inhibition of viral protein (VP-1) synthesis
	Duck Enteritis Virus	1. Inhibition of pro-inflammatory mediators (IL-1α, IL-6, and TNF-α), chemokines (CXCL10 and CCL4) secretion 2. Suppression of NF-$\kappa\beta$ and interferon regulating factor (IRF-3)	1. Inhibition of viral replication, protein synthesis *in-vitro*. 2. Reduction of cellular oxidative damage.
	Human Metapneumonia Virus	1. Suppression of proinflammatory mediators (IL-1α, IL-6, and TNF-α) and chemokines (CXCL10 and CCL4) secretion 2. Inhibitory effect on NF-$\kappa\beta$ and interferon regulating factor (IRF-3)	1. Inhibition of viral replication 2. Reduction of cellular oxidative damage and oxidation stress
	African Swine Fever Virus	Inhibition of protein synthesis and virion formation	Inhibition of viral replication *in-vitro*.
	Human Rhinovirus	1. Suppression of HRV-induced expression of ICAM-1 2. Inhibition of IL-6, IL-8, and RANTES secretion.	Anti-inflammatory effect
	Cytomegalovirus	Inhibition of activated epidermal growth factors (EGF), phosphatidylinositol-3-kinase signal transduction, NF-$\kappa\beta$ and Sp1 transcription factors	Inhibition of HCMV replication and viral protein synthesis *in vitro*
	Polyomavirus	Blocking of DNA synthesis in a dose dependent manner	Inhibition of viral replication *in-vitro*
Quercetin (6, 12)	Duck Enteritis Virus	Along with Resveratrol, it suppressed proinflammatory mediators (IL-1α, IL-6, and TNF-α) and chemokines (CXCL10 and CCL4) secretion	Lowering of cellular oxidative damage
	Human Metapneumonia Virus	Along with Resveratrol it inhibits secretion of pro-inflammatory mediators (IL-1α, IL-6, and TNF-α) and chemokines (CXCL10 and CCL4)	Reduction of cellular oxidative damage
	Herpes simplex virus type 1 (HSV-1)	Along with TNF, quercetin increases the activity of IFN-β and up-regulates the IFN-β Production	Potentiates the dose dependent inhibitory effect of TNF on viral replication.

(Continued)

TABLE 1 | Continued

Name	Virus	Mechanism of action	Effect
	Vesicular stomatitis virus (VSV)	Along with TNF, quercetin increases IFN-β activity and up-regulates the production of IFN-β.	Potentiates the dose dependent inhibitory effect of TNF on viral replication
	Encephalomyocarditis virus (EMCV)	Along with TNF, quercetin increases the action of IFN-β and up-regulates the production of IFN-β.	Potentiates the dose dependent inhibitory effect of TNF on viral multiplication
	Parainfluenza virus type 3 (Pf3)	Inhibits the DNA replication *in-vitro*	dose-dependent reduction in the infectivity of virus
Curcumin (7, 8)	Herpes simplex virus type 1 (HSV-1)	Down regulation of the immediate early (IE) genes.	Inhibition of HSV-1 replication
	Human Immunodeficiency Virus (HIV)	Obstruction of HIV-1 LTR-directed gene expression, Tat-assisted transactivation (Tat protein acetylation) of HIV-1 LTR, HIV-1 and HIV-2 proteases, HIV-1 Integrase	Inhibition of proviral DNA formation, functional protein formation from viral polyprotein and integration of proviral DNA into host DNA
	Influenza Virus	Inhibition of NF-κβ signaling	Inhibition of hemagglutination and viral propagation
	Hepatitis B virus	Increase in the level of p53	Inhibition of viral DNA replication
	Hepatitis C virus	Inhibition of the Akt-SREBP-1 pathway	Inhibition of viral DNA replication
	Coxsackievirus	Dysregulation of ubiquitin-proteasome system (UPS)	1. Inhibition of viral DNA replication, RNA expression 2. Protection against virus-induced apoptosis and cytopathic activity
	Japanese encephalitis virus (JEV)	1. Modulation of cellular levels of stress-related proteins and restoration of membrane integrity 2. Reduction of pro-apoptotic signaling molecules and ROS at cellular level	Provides neuroprotective effect
	Adult T-cell leukemia (ATL)	1. Suppression of DNA binding, transcriptional effect of AP-1 in HTLV-1-infected T-cell lines and JunD protein expression	1. Induction of cell cycle arrest and apoptosis 2. Inhibition of HTLV-1 replication in infected T-cell
EGCG (8)	Influenza Virus	The active blocking of nucleo-cytoplasmic translocation of viral ribonucleoproteins in MDCK cells	Dose dependent inhibition of virus
	Human Immunodeficiency Virus (HIV)	Inhibition of α-glucosidase.	Decrease the infectivity of virus
	Hepatitis C virus (HCV)	Inhibition of NS3/4A protease	1. Inhibition of virus maturation 2. Decrease in pathogenicity
	Herpes simplex virus (HSV)	Along with TNF, quercetin increases the action of IFN-β and upregulates the production of IFN-β	Potentiates the dose dependent inhibitory effect of TNF on viral replication.
	Enterovirus (EV)	1.Inhibition of Viral DNA replication in G6PD-deficient cells. 2. Reduction of EV associated cellular oxidative stress	1. Inhibition of infectious progeny virion formation. 2. Decrease of viral propagation
NAC (9, 13)	Pneumococcal meningitis	1. Scavenging Reactive Oxidation Species 2. Inhibition of inflammatory cytokines	1. Prevention of intracellular oxidation stress. 2. Prevention of Viral Pathogenesis
	Hepatitis C virus (HCV)	1. Scavenging Reactive Oxidation Species 2. Inhibition of inflammatory cytokines	1. Prevention of intracellular oxidation stress. 2. Decrease in viral pathogenesis
	Swine flu (H1N1) virus	1. Inhibit the down regulation of pulmonary catalase, glutathione and superoxide dismutase 2. Scavenging Reactive Oxidation Species	1. Prevention of intracellular oxidation stress. 2. Prevention of Viral Pathogenesis
	Bird Flu (H5N1) virus	Inhibition of the pro-inflammatory cytokines (e.g., TNF-α), chemokines (e.g., IP10) secretion from primary human macrophages *in –vitro*	1. Prevention of intracellular oxidation stress. 2. Prevention of Viral Pathogenesis
	Human Immunodeficiency Virus (HIV)	1. Scavenging Reactive Oxidation Species 2. Deactivation of cellular transcription factor (NFK-β) 3. Inhibition of the upregulation of pro-inflammatory cytokines (e.g., tumor necrosis factor-a) secretion and HIV-1 LTR-directed gene expression	1. Prevention of intracellular oxidation stress. 2. Prevention of Viral Pathogenesis 3. Inhibition of HIV-transcription and replication
PEA (10, 14)	Influenza and common cold	1. Inhibition of the like TNF-α, IL- 1, IL-6, and IL-10. 2. Inhibit adhesion molecules (ICAM-1, P-selectin) and NF-κB expression	1. Prevention of Viral Pathogenesis 2. Alleviation of the symptoms

Certain nutraceuticals have inhibitory effects against a range of viruses in animal and human studies. With the advent of a novel coronavirus strain, nutraceuticals pose as a safer and an efficient substitute to help in offering the relief in those infected with encapsulated RNA viruses.

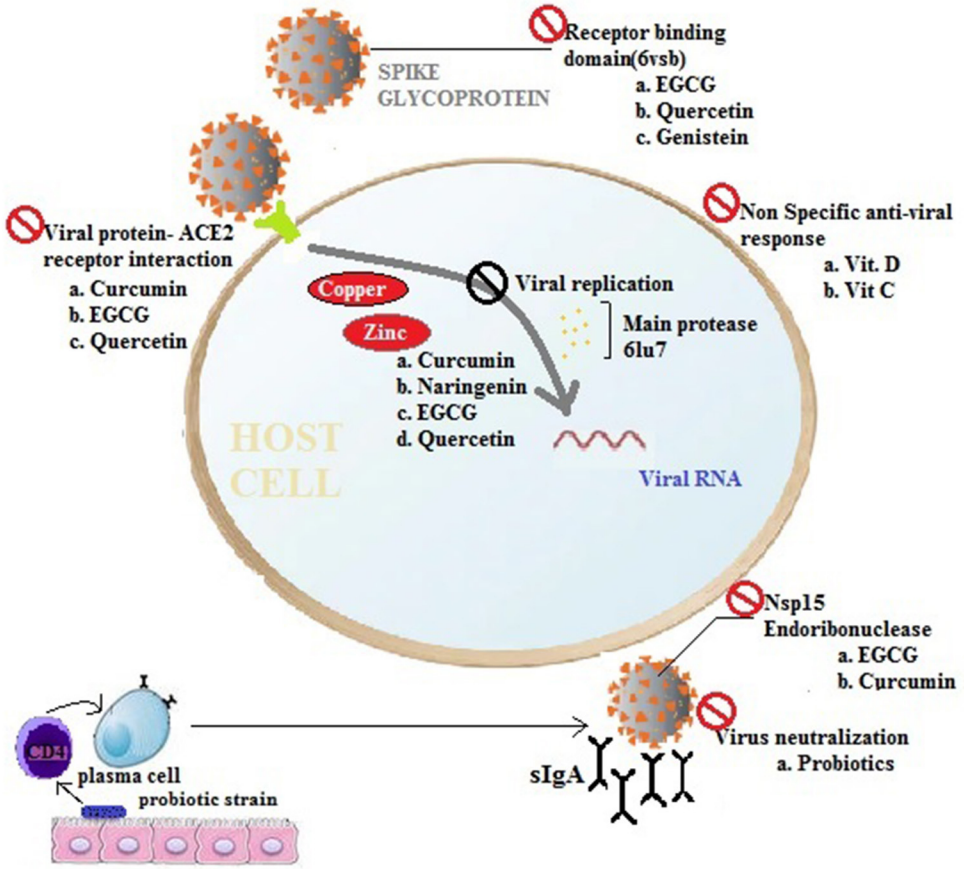

FIGURE 1 | The potency of nutraceuticals and functional foods for preventing viral infections. Pre-intervention as supplements or in combinations with nutraceuticals and nutrient supplements modulate immune responses and prevent viral infections or ameliorate disease severity by acting at different stages of a virus invasion pathway (Nsp15, SARS-CoV-2 endoribonuclease; 6vsb, 2019-nCoV spike glycoprotein; 6lu7, SARS-CoV-2 main protease, ACE2, receptor for SARS-CoV-2; EGCG, Epigallocatechin gallate).

effective against both DNA (e.g., herpesvirus) and RNA (e.g., coronavirus and influenza) viruses. Quercetin can inhibit the viral DNA replication and can also affect the postviral healing by interacting with signaling pathways associated with post-transcriptional modulators. A few studies showed that quercetin in combination with resveratrol suppressed the secretion of pro-inflammatory mediators and chemokines in duck enteritis virus and human metapneumonia virus-led infections, thus minimizing cell oxidative damage (6). A study demonstrated the effectiveness of quercetin in hindering the replication cycle of parainfluenza virus-type 3 (Pf3) by inhibiting its DNA replication *in vitro* (12).

Curcumin

Curcumin (diferuloylmethane) is a polyphenolic group of nutraceuticals, which can be easily obtained from the rhizome of turmeric (*Curcuma longa*) in an abundant amount. Turmeric has been already used as a traditional medicine in Indian and Chinese civilization. In recent times, curcumin becomes a compound of interest to the scientists due to its potential medicinal effects. It is a highly pleiotropic molecule and has well-known antitumor, antioxidant, hypoglycemic, wound healing, anti-inflammatory, antiviral, and anti-infectious properties and further research is still going on (7, 16). The molecular docking method demonstrated that curcumin binds to the target receptors, which are involved in virus infection mechanisms like spike glycoprotein-RBD, PD-ACE2, and SARS-CoV-2 protease, thus blocking virus entry and budding. The study also revealed that curcumin could possibly block ACE2 (a cell receptor, which binds to SARS-CoV-2 spike glycoprotein) to suppress novel coronavirus entry to the cell. Direct incubation with curcumin is said to reduce the ability of enveloped viruses to infect the cells as the former binds to viral surface glycoproteins and inhibits their activity (17). In another study, curcumin administration (50 and 150 mg/kg) *via* oral gavage in an animal model *in vivo* reduced influenza A virus (IAV) replication and lung injury (18). So far, ∼300 clinical trials have indicated toward the usefulness of curcumin against cardiovascular, neurological, cancer, liver, metabolic, pulmonary, and inflammatory diseases. Coronavirus-induced "cytokine storm" results in a multi-organ

failure. Curcumin blocks the necessary regulatory signals that are involved in several pro-inflammatory cytokines expression such as MAPK and nuclear factor-κB (NF-κB) pathways. Curcumin prevents inflammation and lung fibrosis by lowering the expression of vital cytokines and chemokines (IFNγ, MCP-1, IL-6, and IL-10) which are involved in viral infection (19). Despite several health promoting benefits, unstability and low bioavailability *in vivo* are the main factors, which limit the use of curcumin for clinical use on a wider scale. However, the use of other curcuminoids (demethoxycurcumin and bisdemethoxycurcumin), curcumin derivatives and synthetic curcumin analogs, liposome-encapsulated curcumin, curcumin-loaded apotransferrin nanoparticles, and nanoemulsions have increased cellular uptake, solubility, stability, and biological activity (18).

Epigallocatechin Gallate

Flavonoids have been proven to be a useful functional medicine against a number of diseases. EGCG is a common catechin flavonoid, which can easily be found in tea and tea products. EGCG and its esters have encompassed several activities like anti-inflammatory, antibacterial, antiviral, antidiabetic, antihypertensive, etc. EGCG has reported to be helpful against a variety of viruses such as HSV, HCV, enterovirus (EV), and HIV. Besides this, a study demonstrated the capability of EGCG to act against influenza virus by blocking the nucleo-cytoplasmic movement of viral ribonucleoproteins in Madin–Darby canine kidney (MDCK) cells in a dose-dependent approach. (8) It has been hypothesized that EGCG, a zinc ionophore with substantially lower toxicity, can provide a positive effect similar to chloroquine (CQ) by increasing intracellular Zn^{2+} concentration, thus mediating its antiviral effect against SARS-CoV-2. However, human clinical trials need to be done to support *in vitro* studies and to establish the efficacy of EGCG (20).

N-Acetyl Cysteine

N-acetyl cysteine is a prodrug, which is primarily employed as a mucolytic medium and also in the control of acetaminophen poisoning. The antioxidant and anti-inflammatory effects of this compound play an essential role in antiviral activity. Though the mechanism of action of the antiviral activity of NAC is still not fully discovered at a molecular level. However, it has been established that it is not only active against viruses like HIV and other viruses having a similar replication mechanism (which are dependent on nuclear transcription factors for their infectivity) but also against other viruses having a complete different pathogenesis of human diseases. It is a well-known fact that the lack of oxygen in a host cell environment, increased cellular stress due to the absence or less amount of GSH and more free radicals, secretion of inflammatory signal molecules play an important part in the virus pathogenesis, and like a GSH-replenishing prodrug, it helps body to fight against those viruses (9).

Palmitoylethanolamide

Palmitoylethanolamide can be procured from the plant as well as animal sources. It is a cannabinoid receptor-inactive endocannabinoids (eCB)-related molecule, which is mainly used in prophylaxis for helping in the prevention of respiratory viral infection. It is recognized for its regulatory activity in cellular and metabolic homeostasis, antioxidant, anti-inflammatory, and immune-modulating capabilities (15). Its anti-inflammatory and antioxidant properties help in promoting an antiviral effect on the different types of viruses, especially common cold and influenza. It has also been used in Spain and Italy under the brand name Normast and in the earlier Czechoslovakia under the brand name Impulsin till 2008 while it is currently known as PeaPure. In the USA, it is sold as Recoclix for inflammatory bowel syndrome. It is also effective against various autoimmune disorders, like inflammatory diseases of the CNS and inflammatory bowel disease (10). An early randomized controlled trial (RCT) conducted on 468 healthy adults demonstrated that PEA administration lowered (45.5%) the incidences of headache, fever, and sore throat in comparison to the placebo group ($p < 0.05$). In a prophylactic trial with 918 participants, sickness days were reduced by a decrease of 40 and 32% after 6 and 8 weeks, respectively, relative to placebo ($p < 0.0005$). In another trial (901 volunteers) of postoral administration of PEA, a notable decrease in acute respiratory diseases (22.7%) and influenza virus titers was observed to be 34.4% in the placebo group ($p < 0.0002$) (14).

POTENTIAL NUTRIENTS FOR PREVENTION OF VIRAL INFECTIONS

There are several natural compounds available, which have shown an antiviral potential in averting or/and debilitating viral diseases or for therapeutic applications. A nutrient-rich diet can lessen the possibility of chronic diseases and helps in making many viral infections less severe. Nutrients collectively comprise highly potent vitamins (vitamins A, D, C, E, B_6, and B_{12}), minerals (calcium and magnesium), trace elements (zinc, copper, selenium, etc.), carbohydrates, proteins, fats, and water. These multi-nutrients provide the highest nutritional value for all systems of the body, including bone, cardiovascular, liver, skin, and immune support. Therefore, eating a balanced diet rich in multi-nutrients can improve immunity in addition to maintaining respiratory and pulmonary health.

Role of Vitamins in Antiviral Immunity

Vitamins brace the immune system of the body at three different levels, i.e., physical barrier (such as mucous membrane and skin), antibody production, and cellular immunity. Vitamins C and E help in strengthening the physical barriers. Vitamins C, D, and E assist immune functions at a cellular level. In addition, vitamin C is involved in antibody production. **Table 2**, adopted from Wikefeldt (21), provides a general summary of the common sources of nutrients along with their role in body functions, which have been further discussed in the following sections.

Vitamin A (Retinol)

Vitamin A is also recognized as an anti-inflammatory vitamin due to its crucial role in promoting the response of the immune system. It plays a regulatory role in the humoral and cell-mediated immunity through surface IgA, T helper cell

TABLE 2 | Functions of immunity building nutrients in the human body along with their food sources.

Nutrient	Function	Food Sources
Vitamin A	Helps in maintaining mucosal lining of the gastro-intestinal and respiratory tract, regulates innate immunity, production, growth and differentiation of antibodies and lymph cells, anti-inflammation vitamin, inhibit apoptosis.	Orange and yellow fruits, Citrus fruits, sweet potatoes, carrots, bell pepper, dark green leafy vegetables etc.
Vitamin B6	Helpful in fighting against infection by supporting various biochemical reactions in the body, involved in nerve function and antibodies production, communicative interactions between cytokines and chemokines	Whole grains, beans, avocados, sunflower or sesame seeds, pistachios, fish, carrots, fish, milk, rice, and onions
Vitamin C	Antioxidant, protects cells from damage, besides boosting bone and tissue growth, helps in proper working of the immune system by regulating activity of T-lymphocytes and phagocytes, regulates drug metabolization.	Citrus fruits, papaya, spinach, kale, Brussel sprouts, broccoli, tomato, cabbage, cantaloupes, green peas, green, and red pepper.
Vitamin D	Needed for healthy bones, muscles and nerves fibers, regulates innate adaptive immune system response to identify and destroy pathogens.	Fatty fish, egg yolk, liver, mushrooms, fortified milk, juice or cereal, and sunlight (for synthesis)
Vitamin E	Antioxidant, protector of proteins and membrane fatty acids, modulate host immune functions, regulates humoral, and cell-mediated immunity.	Sunflower and safflower seeds, avocados, squash, almonds, peanuts, spinach, tomato, kiwifruit, trout, shrimp, olive oil, wheat germ oil, and broccoli
Protein	Building blocks and required in the production of antibodies and complement proteins	Low-fat dairy products, milk, yogurt, and cottage cheese, beans, brown rice, soy products, nuts, beans, chia seeds, chickpeas, peanuts

As per WHO, nutrients are vital for disease prevention, management of health conditions, growth, and good health. Majority of nutrient quota for the body is met from the food, which comprises fruits and vegetables (21).

modulation, and the generation of cytokines. Vitamin A has shown a proven therapeutic significance in the therapy of several infectious diseases such as measles-associated pneumonia. The supplementation of vitamin A has been widely researched as a part of the potential supporting the therapy for the prevention against the occurrence of acute lower respiratory tract infections (ALRTIs) and reducing the severity and for a speedy recovery. It has been found that children suffering from the deficiency of vitamin A tend to be at a larger risk of death and illness because of respiratory tract infections (15). Furthermore, a meta-analysis study showed that the infection worsened in children with preexisting vitamin A deficiency and its supplementation has been displayed to lower the risk of death by ∼23–30% in 6–59-month old children. Vitamin A can be taken from orange/yellow fruits and vegetables. Permissible dose, as suggested by researchers, is up to 10,000–25,000 IU/day (22).

Vitamin D (Ergocalciferol)

Vitamin D is a steroid hormone and an immune system modulator, which lowers the expression of inflammatory cytokines in addition to increasing the macrophage activity. It also promotes the expression of antimicrobial peptides (AMPs) that are present in natural killer cells, monocytes, neutrophils, and epithelial cells lining the respiratory tract (23). Vitamin D acts both by suppressing and defending against infection by increasing anti-pathogen peptides. A few studies suggest the role of vitamin D supplementation in preventing infections in the upper respiratory tract. It also modulates transforming growth factor-beta (TGF-β) and reduces cytokine expression, thus favorably modulating virus-induced pathological cellular processes (24).

However, the research is limited to laboratory scale, is not established clinically, and shows that vitamin D (increased IL-1β in cell culture) plays an essential role in fighting viral infections. The study suggested that a range of >50 and <80 ng/ml serum 25-hydroxy vitamin D might prove helpful in mitigating morbidity from COVID-19. Current reviews recommend that vitamin D modulates innate responses of the immune system to respiratory viral infections, like RSV, parainfluenza 1 and 2, and influenza A and B (15). Martineau et al. (24) performed an RCT on 11,321 different people of 14 countries and noticed that vitamin D intake significantly lowered the instance of respiratory infections in people already having deficiency besides lowering infection risk in those with sufficient levels of vitamin D. Another study demonstrated the use of vitamin D in improving the response to antiviral treatments in patients suffering from HIV and hepatitis C (25). A study conducted by the Journal of the American Geriatrics Society (2016) showed that elderly patients who were given significantly higher doses of the Vitamin D3 had 40% fewer chances to attain lung infections. Deaths in older people are higher due to infections like bronchitis, pneumonia, and influenza because of their weakened immune function. According to research, vitamin D helps in strengthening the first line of defense with age, thus preventing chronic respiratory infections. About 1,000–4,000 IU of vitamin D/day intake is enough though people with severe deficiency need much higher doses (26).

Vitamin C (Ascorbic Acid)

Vitamin C assists several activities of both innate and adaptive immune system at a cellular level. It gathers in the phagocytic cells of the immune system, like neutrophils, and promotes microbe killing through chemotaxis, phagocytosis, the production of reactive oxygen species (ROS), and finally microbial killing. Vitamin C supplements hold the potential to prevent and cure systemic and respiratory infections by strengthening immune functions and therefore are actively used in hospitals to treat SARS-nCoV-2 infection too. Permissible dose, as suggested by researchers, is up to 1–3 g (one tab by mouth once a day) (27). Prophylactic approaches to prevent infection emphasizes daily dietary intake of vitamin C, which

provides enough if not saturating plasma levels (100–200 mg/day). On the other hand, for the treatment of developed infections, a much higher dose (g) is required to recoup for a higher metabolic requirement (28). A scientific work in 11,306 people comprising 29 studies showed that daily vitamin C supplementation at a dosage level of 1–2 g/day decreased the interval of cold by 14% in children and 8% in adults (29). Furthermore, intravenous vitamin C when administered with high doses improved symptoms in people suffering from serious infections, viral infections-induced sepsis, and acute respiratory distress syndrome (ARDS) (30). However, at higher doses, vitamin C daily can lower the level of copper in the body, especially in people with a copper deficiency, which can in turn adversely affect immune function. A recent study posted to clinical trials by Peng (31) of Zhongnan Hospital, China stated the conduction of vitamin C infusion treatment for the therapy of serious 2019-nCoV infected pneumonia on 140 patients. Another randomized controlled experiment was recorded lately in the Chinese Clinical Trial Registry and conducted on Vitamin C and COVID-19 signifying the importance of vitamin C tablets in combination with diammonium glycyrrhizinate enteric-coated capsules in the treatment of novel coronavirus-caused pneumonia (32). Coronavirus (2019-nCoV) infection induces a cytokine upsurge, which leads to excessive inflammation and consecutively collateral lung damage and higher mortality. Conclusively, vitamin C infusion, an antioxidant, may be used as a symptomatic supportive treatment to help fight against oxidative stress and inflammation. A meta-analysis represented that the length of mechanical ventilation was shortened by 14% in the group that received vitamin C infusion. Several trials on humans, animals, and cells have confirmed the antiviral potential of vitamin C. Furthermore, vitamin C has shown promising results in controlled trials, by lowering blood pressure, decreasing bronchoconstriction, improving endothelial function, lowering the incidence of atrial fibrillation, evading pain, shortening the span of colds, and their incidence in physically worn out adults in addition to having potential beneficial effects against pneumonia (29).

Vitamin E (α-Tocopherol)

Vitamin E is an antioxidant with the potential to regulate the host immune response, and its insufficiency is known to hamper humoral and cell-mediated immunity. A study in elderly patients showed that vitamin E supplementation (200 IU/day) did not have much impact on lower respiratory tract infection but offered a shielding effect on upper respiratory tract infections, in particular common cold (33). The positive effects of vitamin E supplementation positively affected the medication of chronic hepatitis B observed in a small pilot randomized control trial, where a significantly higher normalization of liver enzymes and hepatitis B virus-DNA negativization were noticed in the vitamin E group. Similiar results were noted in a RCT in pediatrics, wherein vitamin E treatment led to a higher anti-HBe seroconversion and virological response (34). Although one RCT depicted that neither everyday multivitamin-mineral supplement nor vitamin E (200 mg/day) intake depicted a beneficial result on the occurrence and severity of acute respiratory tract infections in well-nourished adults. On the other hand, vitamin E supplementation showed increased severity and symptoms, illness duration, and activity restriction in the group (35).

Vitamin B Complex

Vitamins falling under the B complex group play an important role in the proper functioning of the human body including an improvement in the respiratory function activation of the innate and adaptive immune responses and maintenance of endothelial integrity. The administration of high doses of vitamin B_1 or thiamine in patients at early stages of COVID-19 facilitates antibody responses and has the potential of limiting hypoxia. A study on combination of vitamin B_2 (riboflavin) and UV light not only reduced the level of SARS-CoV-2 in human blood but also acted against MERS-CoV virus (36). Vitamin B_3 (niacin) acts as a precursor of NAD and NADP, which acts by reducing pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) and also possesses immunomodulatory properties. It reduces the inflammation in a patient suffering from a ventilator-induced lung injury. Moreover, niacin also prevents the replication of viruses such as vaccinia virus, HIV, hepatitis B virus, and EV. The deficiency of vitamin B_6 or pyridoxine leads to immune dysregulation. A few studies show that low levels of pyridoxine have been observed in COVID-19 patients with high inflammation. Another study on vitamin B_9 or folic acid showed that it was capable of inhibiting the enzyme, furin, thus preventing binding by the SARS-CoV-2 spike protein ultimately to hinder cell entry and virus turnover (37). Similarly, a recent work on vitamin B_{12} (cobalamin) suggested that an intake of methylcobalamin possesses the ability to lower the risk of COVID-19-associated organ failure. Intramuscular administration of the methylated form of cobalamin in patients suffering from its deficiency dramatically restored CD8+ lymphocyte production and increased CD4/CD8 ratio, CD3-CD16+, and CD16 CD57 count in turn boosted the NK cell activity. Further, the reduced level of vitamin B_{12} has also been reported in COVID-19 patients (38).

Role of Trace Elements and Minerals in Antiviral Immunity

A general summary of conventional sources of minerals and trace elements along with their role in body functions is discussed in the following sections as listed in **Table 3**.

Zinc

Zinc acts by favorably modulating an innate and adaptive immune response and virus supported pathological cellular activities through attachment and multiplication. Zinc deficiency is common, and its supplementation is proven to prevent both the duration of viral infections and their severity. Zinc lessens the possibility of lower respiratory tract diseases, which might be of use as far as COVID-19 is concerned. Researchers suggest an intake of zinc (30–60 mg/day) in the form of citrate, glycinate, zinc acetate, orally and zinc gluconate as lozenges (43). Zinc supplements display mitigating effects against several common cold viruses and might also be helpful for patients who are already

TABLE 3 | Functions of immunity building trace elements and minerals in the human body along with their food sources.

Name	Function	Food sources	References
Zinc	Imperative in wound and scar healing, neurocognitive health, development of innate and adaptive immune response against invading viruses and bacteria.	Shellfish, toasted wheat germ, spinach, cashews, pumpkin, sesame seeds, squash, baked beans, chickpeas, dark chocolate	(39)
Copper	Required for maintaining nerve cells, lung elasticity, metabolism, making red and white blood cells, development and differentiation of immune cells	Oysters, organ meat, nuts, seeds, shitake mushrooms, lobster, liver, yeasts, black pepper, potatoes, leafy greens, and dark chocolate.	(40)
Selenium	Protects against oxidative damage and infection, thyroid gland functioning, key nutrient in counteracting virulence development, enhances vaccine responsiveness, development and differentiation of immune cells, delayed-type hypersensitivity activity	Milk and other dairy products, nuts, sea foods, organ meats, cereals, and grains	(41)
Magnesium	Regulates biochemical reactions, glucose levels, protein synthesis, neurological and muscle functions, regulates innate immunity, anti-inflammatory, antibody synthesis.	Almonds, spinach, roasted cashews, peanuts, soy milk, avocado, brown rice, yogurt, beans, banana.	(42)

Like vitamins, minerals and trace elements are classified as nutrients, which support several bodily functions, including immunity optimization, and energy production. Antiviral properties of some of the minerals (magnesium) and trace elements (zinc, copper, and selenium) have been backed with strong scientific evidence.

ill. One of the studies conducted in 64 hospitalized children suffering from ALRTI showed that the administration of 30 mg zinc daily lowered the infection duration by ∼2 days as compared to the control group (44). Moreover, long-term zinc intake is considered generally safe for healthy adults (set upper limit, 40 mg). On the contrary, excessive prolonged doses may obstruct the absorption of copper, thus compromising the immune system. Many *in vitro* and clinical studies signify the effectiveness of zinc in eliciting antiviral activity. Shida (45) demonstrated that zinc had a strong effect on numerous respiratory viral infections by the modulation of the entry of viral particles, replication, fusion, viral protein translation, and its release. In another study, low Zn concentration increased the susceptibility to pneumonia, and subjects having a high serum Zn level (>70 μg/dl) were considered to be at a lower risk of getting pneumonia ($p < 0.001$), along with lower mortality and disease duration in comparison to the low-zinc group (<70 μg/ml). In addition, serum zinc concentration was reported to be 15% lower in the incidences of community acquired pneumonia (46). Zinc ions might have an anti-inflammatory activity in pneumonia, thereby minimizing oxidative stress and protecting the lungs against damage in sepsis and systemic inflammation. Zinc deficiency in the murine model of polymicrobial sepsis led to higher NF-κB p65 messenger RNA (mRNA) expression in lungs, which caused the upregulation of the target genes of TNFα, IL-1β, and ICAM-1. On the other hand, taking zinc supplements produced a protective effect in the lungs in the septic state with the help of the modulation of NF-κB and ERK 1/2 by lowering neutrophil infiltration and oxidative damage (47). The observed study also displayed that zinc stops the activity of RNA-dependent RNA polymerase (RdRp) of hepatitis E virus, ultimately affecting its replication. Zinc reportedly inhibits coronavirus RdRp activity as well *in vitro* and zinc ionophores to restrict coronavirus replication. Even though the antiviral properties of zinc have been well-established in addition to the possible property of CQ/hydroxychloroquine (HCQ) acting as a zinc ionophore, the synergistic effects of zinc with any one of these drugs still need to be confirmed. It is being speculated that high intracellular zinc concentration might also cause more proper RdRp obstruction resulting in more hinderance of intracellular SARS-CoV-2 multiplication (48).

Copper

Copper aids in the development and differentiation of the cells of the immune system. *In vitro* studies have also depicted the antiviral properties of copper. Copper can kill various viruses such as HIV-1, bronchitis virus, poliovirus in addition to other enveloped and non-enveloped, and double- and single-stranded DNA and RNA viruses. Virus killing by copper might be mediated through ROS (49). Intracellular copper has been proven to regulate the influenza virus life cycle while copper-thujaplicin complex inhibits the replication of human influenza viruses. Turnlund et al. (50) studied the impact of prolonged high copper consumption on the immune system function in young men. They concluded that benzylamine oxidase, superoxide dismutase, and plasma ceruloplasmin activity were more in amount when the copper intake was 7.8 mg/day, as compared to an intake of 1.6 mg/day, thus showing an enhancement in the antioxidant level (50). Though a significant reduction in the percentage of serum IL-2R, circulating neutrophils, and the antibody titer was observed at a higher copper intake (7.8 mg/day) against the Beijing strain of influenza (4). The literature also illustrated that the exposure of copper to nCoV 229E irreversibly affected the morphology of virus by breaking it into envelope and dispersing surface spikes in addition to destroying the viral genome (51). A cell-based study proved that copper ions were able to block the protease-2 required by SARS-CoV-1 for its replication. Another study conducted in China on 71 adults suffering from COVID-19 found that all participants had a relatively low serum total cholesterol level in comparison to healthy adults. However, several studies have also indicated the link between a low level of total cholesterol to the reduced concentration of copper (52). Thus, copper supplements might also prove helpful in the fight against novel viral diseases like COVID-19 through extensive clinical

data, and experimental results may be required to support the same.

Selenium

Selenium is an important mineral for a healthy immune system. Research on animal models indicates the potential of selenium supplementation in increasing the antiviral response against various influenza strains, such as H1N1. A few studies on the virulent strains of influenza and coxsackie viruses demonstrate that the acute deficiency of selenium can enhance disease severity and pathogenicity by promoting numerous mutations in the viral RNA. Therefore, selenium is essential both for boosting host antiviral (Th1-type) immunity and for obstructing the evolution of some viral pathogens into more virulent strains. Reduced concentration of selenium and selenoenzymes [such as thioredoxin reductase (TrxR) and glutathione peroxidase (GPx)] in erythrocytes and plasma has been observed in children afflicted with highly virulent H1N1 subtype of IAV (53). In a French RCT conducted on adults having low plasma selenium levels were given 20 mg Zn and 100 mg Se supplements for 15–17 months. It was found that adults receiving supplementation displayed a better humoral defense post-influenza A vaccination in comparison to the adults from the placebo group (54). IAV-infected tissues and cells may be safe guarded from virus-led oxidative stress and cell death with the optimized activity of GPx under the conditions with adequate selenium. Also, according to a study, bronchial epithelial cells cultured in a Se-deficient environment showed more cell death due to apoptosis after being infected by IAV in comparison to cells grown in Se-adequate medium (55).

Magnesium

Magnesium is vital in regulating the immune function by exerting an influence on antibody synthesis, antibody-dependent cytolysis, immune cell adherence, immunoglobulin M lymphocyte binding, T helper-B cell attachment, and the response of macrophage to lymphokines. It acts as both an anti-inflammatory and a bronchodilator and has been used in a successive manner to clear the airways and make it easier to breathe. Some *in vivo* and *in vitro* studies emphasize upon the importance of magnesium supplementation in developing the immune reaction against viral infections (56). One of the studies showed that reduced concentration of free intracellular Mg^{2+} is responsible for an impaired expression of receptors of CD8+ T cells and natural killer cells (NKG2D) which in turn affects the cytolytic action against viruses and immune surveillance (6). Two patients suffering from EBV were given oral supplements of magnesium for 175 days in the form of magnesium threonate and magnesium sulfate, on patients suffering from severe EBV infection. Within 2 days of administration, an increase in NKG2D expression and free [Mg^{2+}] ions was observed in patients along with a decrease in the number of EBV-infected cells. This implies that [Mg^{2+}] ion homeostasis in the body is essential for NKG2D expressed CTLs, NK cells, and γδ T cells, which in turn mediate antiviral and antitumor immunity (56).

MULTI-NUTRIENT SUPPLEMENTS FOR VIRAL INFECTIONS

The deficiency of micronutrients in the body weakens the immune system by affecting the adaptive antibody response and T-cell-mediated immune response, subsequently causing a balanced host response dysregulation. Certain trace elements and vitamins support immunity by strengthening epithelial lining as well as cellular and humoral immune responses. Moreover, trace elements and vitamin supplement in various combinations have shown favorable results on the antiviral ability of immune response. In a randomized clinical trial comprising 725 elderly patients, considering the parameters such as humoral response to influenza vaccine, delayed-type hypersensitivity skin response, infectious mortality, and morbidity depicted that adequate zinc supplementation in combination with selenium increased humoral activity post immunization relative to the control group. A study on older adults showed that multi-nutrient supplement comprising a combination of trace elements, such as selenium sulfide and zinc, vitamins like ascorbic acid and β-carotene, corrected specific nutrient deficiencies within 6 months of administration. Respiratory tract infections were absent in patients who received trace elements. In addition, it was observed that the level of antibodies after influenza vaccine administration was higher in groups, which received trace elements alone or were associated with vitamins as compared to the group that received vitamin alone with lower antibody titers (54). One study conducted on 878 patients suffering from HIV subtype C having a higher cell count than the normal (>350/μl) and underwent antiretroviral therapy revealed that the supplementation of vitamin E plus selenium, vitamin B complex, and vitamin C helped in slowing down the disease progression in addition to lowering the morbidity (57).

ROLE OF MILK PROTEINS AND PEPTIDES IN ANTIVIRAL IMMUNITY

Protein imbalance in the diet leads to malnutrition resulting in impaired immunity, particularly affecting the T-cell system, leading to increased opportunistic infections and mortality in hospitalized patients. Active milk proteins and peptides possess antiviral and immune regulating characteristics (58). Human and bovine lactoferrin (bLF), lactoperoxidase, artificially altered milk proteins such as serum albumin, β-lactoglobulin, and α-lactalbumin act on viruses like HIV, by binding to their cellular receptors, thus inhibiting viral absorption and ultimately replication. Lactoferrin can effectively bind to the heparan sulfate and mannose receptor of HIV, which inhibits virus attachment (59). Another work demonstrated the usefulness of lactoferrin in strongly inhibiting viral reverse transcriptase. Meanwhile, α-lactalbumin, β-lactoglobulin, and casein were effective in strongly inhibiting the protease and integrase of HIV (60). Lactoferrin of both human and bovine origin has been depicted to show an antiviral response against a wide spectrum of viruses such as influenza virus (H1N1, H3N2, and H5N1) dosage,

esterified bLF <20 mg/ml. The antiviral response of lactoferrin against parainfluenza virus and RSV is also demonstrated by inhibiting viral replication (61). Even the methylated forms of β-lactoglobulin, α-lactalbumin, and lactoferrin elicit the antiviral response against human influenza virus A, H3N2, H1N1, and lethal avian IAV (H5N1). The antiviral response is linked to the binding of whey proteins to virus hemagglutinin, viral DNA and RNA, and is disrupted, thereby making viral proteins unstable and incapable of their attachment to the cell membrane (62). One of the studies indicated that the intake of lactoferrin along with milk immunoglobulin reduces the occurrence of common cold in adults. Lactoferrin intake increases the NK cell response in adenomatous colorectal polyps patients. Therefore, lactoferrin might partially mediate protection to the host against influenza and common cold by increasing the number and activity of NK cells (63). Lactoferrin has also been classified as a host defense protein due to its ability to enhance cytotoxicity by increasing the functions of lymphokine-activated killer cells and NK cells particularly in infants. In addition to this, it is also involved in macrophage activation along with the stimulation of pro- and anti-inflammatory cytokine release IL-1, IL-6, IL-8, IL-18, IL-γ, and TNF-α. Further, a few studies on milk proteins and peptides have demonstrated synergy with drugs such as acyclovir, ribavirin, and zidovudine, against HSV, human HCV, and HIV 1, respectively, by reducing drug dosage, preventing the development of drug-resistant viruses, and selective targeting (64).

FUNCTIONAL FOODS FOR ENHANCING IMMUNITY

Functional food is not a single component; it is a combination of different nutrients that are high in a particular component imparting therapeutic benefits. Functional foods contain supplements or additional nutrient-rich ingredients, such as oats, a rich source of dietary fiber having a beta-glucan, or fiber-enriched vermicelli, which increase the immunity by reducing the inflammation, and thereby helps in improving the health (65). These constitute conventional (grains, fruits, vegetables, fermented foods, herbs, spices, beverages, and nuts) or wholesome natural foods as well as modified foods (yogurt, cereals, and orange juice) that can be fortified with vitamins, minerals, and probiotics for additional health benefits. In July 2002, about 300 food products were recognized as foods for specified health use (FOSHU) status in Japan. Similarly, a large quantity of antioxidants lying in vegetables and fruits also help in combating diseases (66). A few previous literature studies has also shown that fried food has impaired the white blood function and severely altered gut microbiota. Plant-based diets have been proven to be effective in reducing the risks for influenza and pneumonia as these are rich in dietary fiber, antioxidants, and vitamins (67), which lower body mass index (BMI), and thereby help in improving immunity. However, health benefits and claims associated with the consumption of these functional foods still need to be worked upon to establish a strong scientific proof regarding its safety and efficacy. Honey is one such functional food having a well-studied antiviral potential. Apart from sugars, honey contains several other minor components such as minerals, vitamins (majorly vitamin C), carotenoids, proteins, amino acids, enzymes (catalase and glucose oxidase), volatile compounds, and organic acids. The constituents impart several benefits to honey, which is possibly used in the treatment of diseases by anti-inflammatory, immunomodulating, phytochemical, antioxidant, antibacterial, antiviral, antitumor, and vasodilative activities. Flavonoid and phenolic compounds of proven therapeutic significance, such as gallic acid, ellagic acid, cinnamic acid, benzoic acid, caffeic acid, coumaric acid, apigenin, myricetin, quercetin, catechin, naringenin, and luteolin, are the main bioactive compounds present in honey, which also exert an antioxidant potential. Vitamin C and phenolic content together impart an anti-inflammatory effect. Evidence suggests that honey lowers the inflammatory action in cell cultures, animal models, and clinical trials. A tissue culture study indicated that honey enhanced the level of antibodies, T and B lymphocytes, neutrophils, eosinophils, monocytes and the generation of NK cell production during immune response (68). Manuka honey from New Zealand is said to possess antiviral activities against influenza virus (H1N1) strain A/WSN/33 in MDCK cells. Another *in vitro* study also showed the effectiveness of commercial manuka honey against a HSV-1 isolate using Vero cells (69). Charyasriwong et al. (70) reported that an active ingredient, methylglyoxal, present in manuka honey showed an activity against H3N2, H1N1, H5N2, and also oseltamivir-resistant H1N1. Similarly, berries are rich in bioactive compounds, particularly polyphenolics, flavonoids along with polysaccharides, carotenoids, organic acids, anthocyanins, etc. have been used as a natural cure against upper respiratory tract infections. Elderberry extract exerts a regulatory influence on viruses both by directly obstructing viral glycoproteins and indirectly by increasing the expression of IL-6, IL-8, and TNF (71, 72). A concoction of raspberry extract, elderberry juice, and honey, named, Sambucol stopped the hemagglutination and multiplication of 10 different influenza type A and type B virus strains *in vitro*, which were isolated from both humans and animals. The therapeutic effect of Sambucol containing berry extracts may be due to the stimulation of pro-inflammatory cytokines (IL-6, IL-8, IL-1β, and TNF-α) production, and anti-inflammatory cytokine (IL-10) by macrophages as well (73). The administration of goji berries increased the activity of flu vaccine in adult mice in addition to increasing cytokine secretion and IgG titers, thereby modulating the immune response (TNF-α and IL-12). Increased maturity and expression of dendritic cells (CD40, CD80, and CD86) were also observed (74). Another research work reported that a black currant (*Ribes nigrum*) extract obstructed influenza type A and type B virus adsorption as well as the pandemic strain oseltamivir-resistant A/Yamagata/5/2009 and A/Yamagata/165/2009 pdm. Furthermore, a black currant extract also checked the factors involved in influenza infection such as pneumonia causing bacteria (*Streptococcus pneumonia*), *Haemophilus influenza*, and viruses like adenovirus (AdV) and RSV, which infect the respiratory tract (75). Krawitz et al. (76) observed that the administration of Rubini, a

commercial elderberry extract preparation, inhibited the activity of pathogens responsible for respiratory diseases including influenza viruses (type A and type B). Similarly, cranberry juice and its polyphenolic component have been also revealed to inhibit influenza A and B strains lowering infectivity titers of rotaviruses and food-borne viruses like feline calicivirus and murinenorovirus (77). Polyphenolic fractions rich in polyphenolics extracted from bilberry (*Vaccinium myrtillus* L.), Natsuhaze (*Vaccinium oldhamii* L.), cranberry (*Vaccinium oxycoccos* L.), and procyanidin, fraction from Canadian blueberry (*Vaccinium angustifolium* L.) have also exhibited a significant antiviral activity against influenza viruses (78). A study disclosed that an aqueous extract of Korean black raspberry (*Rubus coreanus*) inhibited hepatitis B activity. A similar study on another raspberry species (*Rubus imperialis* L.) depicted anti-HSV1 activity *in vitro* (79). Therefore, berries rich in several bioactive components could be a vital raw material for new drugs. Though, more lab studies and clinical trials need to be undertaken to establish their antiviral efficacy.

ROLE OF PROBIOTICS SUPPLEMENTS IN ANTIVIRAL IMMUNITY

Probiotics are live microorganisms that impart health benefits when provided in a sufficient quantity to the host. Bacteria from the genus *Lactobacilli* and *Bifidobacterium* are the most common probiotic microorganisms. Probiotics can be incorporated in the diet in the form of buttermilk, yogurt, bread, sourdough, tempeh, bread, kombucha, cottage cheese, fermented sauerkraut, fermented pickle, miso soup, and kimchi (80). A research work demonstrated that *Lactobacillus rhamnosus* GG (L. GG, ATCC 53103) is completely safe for consumption even by premature infants, indicating the potential of probiotics as a relatively safer option for therapy-based intervention in any age group (81). However, people possessing a weakened immune system due to chemotherapy and critical illness need to be cautious while using probiotic supplements. Specific probiotics have known to be useful in reducing the severity and duration of acute rotavirus-caused gastroenteritis and virus-originated respiratory tract infections.

Even though vaccines are promising prophylactics that are useful against viruses, but their efficiency is limited due to rapidly mutating viral RNA such as in the case of influenza virus. In this regard, probiotics needs to be widely researched to establish their efficacy as a part of antiviral supportive therapy as they have proven a virucidal action against many respiratory viruses as well (**Table 4**). The mechanisms by which probiotics act against respiratory viruses most likely are as follows: (a) by adhering to the epithelial layer, hence block the adherence of viruses by a steric hindrance; or by competing with them for specific carbohydrate receptors, (b) by directly inhibiting the attachment of virus to host receptor cells by binding to it, (c) likely by inducing mucosal regeneration; intestinal mucins may inhibit viral replication by inhibiting their adherence to epithelial cells, (d) by directly producing antimicrobial substances against pathogens, (e) by activating and modulating the immune response *via* dendritic cells and macrophages, (f) by inducing dehydrogenase and mild NO, lactic acid and dehydrogenase generation may have antiviral activities, (g) stimulating the immune system by IL, NK cells, Th1 activity, and IgA production (3, 89).

TABLE 4 | Effect of different probiotic strains on viruses.

Probiotic strain	Target disease/virus	Mechanism of action
lactobacillus casei (yakult) (82)	Upper respiratory tract infection, Epstein–Barr virus (EBV), Cytomegalovirus (CMV)	Lowered plasma CMV and EBV immunoglobulin titers
lactococcus lactis jcm5805 (l. lactis plasma) (83)	Influenza	Reduction in the duration of cough and sore throat Increment in IFN-α mRNA in PBMCs
lactobacillus rhamnosus gg (84)	Rhinovirus infection	Slight reduction in the incidence and severity of cold symptoms
l. plantarum 06cc2 (3)	IFV A/PR/8/34 (H1N1)	Decrease in body weight, virus count in lungs and number of macrophages and neutrophils in bronchoalveolar lavage fluid (BALF), TNF-α in BALF, INF-α, IL-12, and IFN-γ Increase in activity of NK cell, IFN-γ in Peyer's patches and survival of mice
l. plantarum ncimb 8826 (85)	Pneumonia virus of mice (J3666)	Enhanced protection against virus infection Decrease in Granulocyte recruitment, CXCL10, CXCL1, CCL2, TNF, and virus recovery
l. reuteri f275 (86)	Pneumonia virus of mice (J3666)	Increase in neutrophil deployment, CXCL1, CCL2, CCL3, CXCL10, TNF-α, IFN-α, IFN-β, IFN-γ, and IL17A
Enterococcus faecalis fk-23 (87)	Hepatitis C virus	Significant reduction in alanine aminotransferase No significant difference in viral load
Bifidobacterium animalis (bb12) (88)	Intestinal Ig responses to rota and polio- virus in infants	Evident increment in fecal anti-poliovirus and anti-rotavirus specific IgA.

Probiotics depict the ability to regulate and modulate the immune system. Some probiotic strains have shown an effective antiviral activity including respiratory viruses. However, the mechanism of action against viruses differs from one probiotic strain to another.

Probiotics for Upper Respiratory Tract Infections: Clinical Studies

The role of probiotics in preventing upper respiratory infections (URIs) has been extensively studied.

A specific interaction of probiotics with pathogens can potentially reduce their colonization in the nasopharynx, hence reducing URI and acute otitis media (AOM). The microbiota of an individual could be studied and integrated into healthcare to target his specific diseases for a better treatment. Yogurt consumption consisting of *Lactobacillus delbrueckii*s sp. bulgaricus OLL1073R-1 (R-1) triggered the activity of NK cell

and probability of getting a common cold in the elderly was reduced (82, 89). Similar studies demonstrated the significance of secreted polysaccharides of R-1 in improving immune functions along with NK cell activation. Thus, R-1 or its products might play a role in preventing virus-induced respiratory infections (90). In another study, it was found that intranasal inoculation of *Lactiplantibacillus plantarum* or *Lactiplantibacillus reuteri* guarded against lethal infection caused by pneumonia virus in mice (85, 86). Furthermore, probiotics along with prebiotics have proven their efficacy in increasing immunogenicity by affecting seroprotection and seroconversion rates in the elderly administered with influenza vaccine (87, 91). Therefore, a vitamin-probiotic combination could act as a possible immunity booster in a generic manner. For example, vitamin D can modulate an adaptive and innate immune system as the vitamin D receptor is expressed on the surface of all immune cells and also all immunologic cells can produce a vitamin D metabolite (88, 92).

CONCLUSION

For new viral diseases, no specific pharmacological treatment for their prevention or treatment can be made available immediately. In this regard, research focuses on strengthening the immune system by adopting nutritional strategies. This review aims to put forward the therapeutic and preventive potential of some nutrients, nutraceuticals, trace elements, milk proteins, peptides, functional foods, and probiotics. Further, an awareness about the use of nutrient fortified cereals and grains, nutraceutical products should be created as a part of disease preventive and health promotive approach. In view of the current pandemic of COVID-19, a type of severe acute respiratory infection and a deficit of effective targeted antiviral drugs exist thus a symptomatic support therapy is still the approach to be followed. An in-depth knowledge of the regulatory molecules involved in the molecular mechanism of epigenetic interaction and replication can promote the development of functional food products having an antiviral and immunity potential as a component of the effective therapeutic strategy. Therefore, it is crucial to study the clinical relevance and safety of compounds with proven immune enhancing properties for viral pneumonia as well through RCT. Likewise, zinc ionophores like quercetin and EGCG can act in a way similar to drugs such as CQ/HCQ by increasing intracellular Zn^{2+} levels without adverse effects. Clinical trials and *in vitro* studies can be done in this regard. Milk proteins and related peptides have enormous scope to be used as supplements, templates, and novel vaccine adjuvants for designing further potent antiviral drugs. Bioactive compounds such as nutraceuticals and functional foods with proven efficacy in hindering viral mechanisms, along with pharmaceutical medication in case of not being alone, might be instrumental in treating corona virus-induced infections. Although these supplements are beneficial for the immune system and health, they should not be used as an alternative to a healthy lifestyle, which is of utmost importance. Moreover, nutraceuticals are mostly food than medicine and act gradually, thus their long-term and regular ingestion is imperative in reaping the health benefits completely associated with them.

AUTHOR CONTRIBUTIONS

SS: investigation, visualization, formal analysis, and writing—original draft. PK: investigation, visualization, and writing—original draft. DK: resources, methodology, and visualization. VM: formal analysis, resources, and visualization. GS: resources, methodology, and formal analysis. KM: conceptualization, visualization, and writing—review and editing. PP: conceptualization, supervision, and writing—review and editing. MK: conceptualization, supervision, writing—review and editing, project administration, and funding acquisition. All authors contributed to the article and approved the submitted version.

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